Physiological and behavioural responses to environmental stress in abalone: Why is being a hybrid an advantage?

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Doctor of Philosophy

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The essence of the scientific spirit is to realise what a wonderful world it is that we live in

C. V. Raman

Declarations and statements

Declaration of originality

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KA developed the research idea, conducted the experiment, collected and analysed the data, and wrote the manuscript (proportion of contribution 75%). SJA, TDC, and NGE assisted with the research idea, analysing of the data, and manuscript writing.

Paper 4 (chapter 6): **Alter K**, Andrewartha SJ, Clark TD, Morash AJ, Hellicar AD, Leon RI, Elliott NG (2017) *Aquaculture* **478:** 25-34.

KA developed the research idea, conducted the experiment, collected and analysed the data, and wrote the manuscript (proportion of contribution 70%). ADH and RIL provided guidance for data analyses. AJM, SJA, TDC, and NGE assisted with the experimental design and provided guidance during data collection. All authors revised the manuscript.

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General abstract

Australian abalone aquaculture is facing commercial and environmental challenges that may impact abalone performance and industry profitability. This calls for a more in-depth understanding on how abalone respond to environmental change to ensure that abalone farming remains sustainable. The aim of this thesis was to determine the physiological and behavioural responses of the commercially important Australian abalone species, blacklip abalone *Haliotis rubra*, greenlip abalone *H. laevigata*, and their interspecies hybrid to environmental and farm stressors. The main question centres on the most important commercial abalone, the hybrid, and asks why it has improved growth in comparison to pure parental species.

The life of an abalone on Australian abalone farms begins in the hatchery where biotic and abiotic factors are relatively well-controlled. Yet, the physiological and behavioural responses of early life-stage abalone to rearing conditions are largely unknown. The influence of stocking density, oxygen availability, and light levels on oxygen consumption rate ($\dot{M}O_2$) was tested with fertilised eggs and all larvae stages of hybrids. In addition, in a second study acute thermal preference, swimming speed, and $\dot{M}O_2$ were determined across an ecologically relevant temperature range for veliger larvae of *H. rubra*, *H. laevigata,* and their hybrid. Current farm conditions, i.e. stocking densities, light and oxygen levels had no influence on $\dot{M}O_2$ of early-life stages of hybrids. Thermal preference of all three abalone groups increased during larval development from 16 to 20 °C for early to late veligers, respectively. Veliger $\dot{M}O_2$ increased throughout the temperature range tested in all three abalone groups and $\dot{M}O_2$ of hybrids reached a peak at 25 °C. These results provide support that current hatchery conditions are generally within optimal ranges for early-life performance.

As juveniles, abalone are exposed to uncontrolled environmental conditions, e.g. temperature and oxygen level, in grow-out tanks. Juveniles rely frequently on anaerobic energy production and may increasingly depend on it during exposure to stressful conditions, reducing their potential for growth. I tested whether 15 month old juvenile hybrids have a distinct movement behaviour, and/or differ in their use of aerobic and anaerobic energy sources in comparison to parental species when exposed to various temperatures and oxygen levels. Enzyme activities of tauropine and lactate dehydrogenases were similar between hybrids and pure species. Hybrids tended to have an intermediate movement pattern between

the more active *H. rubra* and the less active *H. laevigata*, and a lower resting $\dot{M}O_2$ in comparison to both pure species. These differences were indicative but not significant to allow support of the hypothesis that hybrids have an energetic advantage over pure species.

Some Australian abalone farmers notice higher mortality rates of larger abalone during summer months, suggesting that abalone may be vulnerable to warming and/or hypoxia. Movement, $\dot{M}O_2$, and heart rate were determined in 22 month old hybrids, *H. rubra*, and *H. laevigata* during acute temperature increase following acclimation to control and typical summer farm conditions. Movement of hybrids and *H. laevigata* was not affected by environmental condition, while *H. rubra* showed a strong thermal response. Heart rate- and $\dot{M}O_2$-temperature relationships of hybrids remained similar irrespective of oxygen level, while pure species adjusted both parameters. Heart rate and $\dot{M}O_2$ maxima of hybrids were more stable than those of both pure species across acclimation conditions. These results support the hypothesis that hybrids are less sensitive to changes in environmental conditions, which may contribute to their growth advantage over long grow-out periods in the abiotically unstable farm environment.

In summary, the studies outlined in this thesis have improved our understanding of physiological and behavioural responses of abalone to the most important environmental factors that govern abalone performance. Hatchery conditions are unlikely to negatively influence abalone performance. Yet, fluctuating temperatures and oxygen levels in the grow-out tanks lead to exposure to environmental conditions near the tolerance limits of abalone during summer months, rendering abalone vulnerable to future environmental challenges. The hybrids are more resilient than the pure species to current on-farm fluctuations, suggesting that hybrids are most suited for the aquaculture environment. The farm environment, however, is complex and abalone are likely to encounter additional stressors during culture which may result in increased metabolic constraints and thus reduced growth potentials. The successful laboratory trial, with heart rate sensors, in this thesis opens the door to future on-farm studies to address how additional stressors influence abalone physiology and underpin aquaculture production and sustainability.

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List of abbreviations selected

% O_2sat	Oxygen level in percent air saturation
AAGA	Australian Abalone Growers' Association
ABT	Arrhenius break-point temperature
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bpm	Beats per minute
BZ	Benzocaine
CAT	Catalase
CO_2	Carbon dioxide
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CT_{max}	Critical thermal maximum
CT_{min}	Critical thermal minimum
CuZnSOD	Copper/zinc superoxide dismutase
DO	Dissolved oxygen
DW	Dry weight
EAT	Effective accumulative temperature
FAA	Free amino acids
FCR	Feed conversion rate
GPx	Glutathione peroxidase
H^+	Hydrogen ions
HCl	Hydrogen chloride
Hcy	Haemocyanin
hpf	Hours post fertilization
HSP	Heat-shock protein
IMAS	Institute for Marine and Antarctic Studies
JTA	Jade Tiger Abalone
MnSOD	Magnesium superoxide dismutase
$\dot{M}O_2$	Oxygen consumption rate
mRNA	Messenger ribonucleic acid
MS	Magnesium sulphate

NaOH	Sodium hydroxide
NH_3	Ammonia
NH_4^+	Ammonium
OCLTT	Oxygen- and capacity-limitation of thermal tolerance
P NMR	Phosphorus nuclear magnetic resonance
PA	Phosphoarginine
P_{crit}	Critical oxygen level
PE	Phenoxyethanol
ROS	Reactive oxygen species
SDNN	Standard deviation in beat duration
SeGPx	Selenated gluthatione peroxidase
SL	Shell length
SOD	Superoxide dismutase
T_{pref}	Preferred temperature
TPx	Thioredoxin peroxidase
TRx-2	Thioredoxin-2
U	Swimming speed
UTas	University of Tasmania
WW	Wet weight

CHAPTER 1: General introduction

Ventral and dorsal views of juvenile abalone used in this thesis: blacklip abalone, *H. rubra* (left), greenlip abalone, *H. laevigata* (centre), and their interspecies hybrid abalone (right).

Biology of southern Australian abalone

Abalone are herbivorous archaeogastropods of the genus *Haliotis*. They are distributed throughout the world with more than 87 species recognized from the tropics to temperate regions (Geiger and Poppe, 2000). In Australia, populations of the commercially important and dominant endemic species, blacklip abalone, *H. rubra* Leach, and greenlip abalone, *H. laevigata* Donovan, occur on shallow reefs along the southern coasts of the country (Shepherd, 1973). Reproductive activity of *H. rubra* is high in late winter, but can occur throughout the year, while *H. laevigata* spawns from spring to autumn (Shepherd and Laws, 1974; C. Mundy, University of Tasmania, pers. comm., October 2015). The abalone release their eggs and sperm into the water where, after successful fertilization, embryos transition to trochophore larvae and then veliger larvae (Heasman and Savva, 2007). These early-life stages are planktonic and lecithotrophic (Shepherd *et al*., 1992). It is not known where early-life development takes place because no embryos and only a few larvae have been found in the wild (Breen and Adkins, 1980; McShane *et al*., 1988; Babcock and Keesing, 1999). Yet, early-life abalone are suggested to have a low dispersal range and develop close to juvenile and adult populations (Prince *et al*., 1987). Juvenile and adult *H. rubra* inhabit sheltered areas of rocks and occur most commonly at water temperatures of 11 to 19 ºC while those of *H. laevigata* are often found on rocks exposed to rough waters and occur most commonly in waters of 12 to 23 ºC (Shepherd, 1973). In areas where the two species live sympatric, a fertile *H. rubra* × *H. laevigata* hybrid can also be found in low abundances (Brown and Murray, 1992; Brown, 1995). The hybrid has an intermediate morphology and thus can be visually distinguished from the pure parental species (Lafarga-De la Cruz and Gallardo-Escárate, 2011; see cover photo of this chapter).

Australian abalone aquaculture

Farming stages

Australian abalone aquaculture began in the early 1980s and has grown steadily ever since. Abalone production increased from 21 T in 1999 to over 900 T in 2014 and is expected to reach 4000 T in 2025 (Li, 2008; Savva, 2015). The majority of the farms are land-based on which abalone pass through three farming stages, namely hatchery, nursery, and grow-out phase (Li, 2008; Savva, 2015). Different cultivation procedures and environmental parameters are recommended to optimise production at the three stages (Heasman and Savva, 2007; Table 1.1).

T**able 1.1**: The three farming stages for land-based abalone aquaculture in Australia. Cultivation practices from Jade Tiger Abalone, in Victoria, are noted.

	Hatchery	**Nursery**	**Grow-out**
Life-stage	Embryo to settling larva	Post-larva	Juvenile and adult
Age	1 to 7 days	1 week to 3 month	3 month to 2.5 years
Feeding	Non-feeding	Grazing on biofilm	Formulated feed
Holding system	500 L tanks	Submerged vertical plastic plates	Concrete slab tank
Temperature	Maintained, 16 to 18 °C	Uncontrolled, commonly 16 to 24 °C	Uncontrolled, 9 to 23 °C
Oxygen	Tanks are aerated, > 90% O_2sat	Tanks are heavily aerated, > 98% O_2sat	Uncontrolled, 100 to < 70% O_2sat

In the hatchery, the embryos and larvae are reared at a recommended stocking density of ~ 20 individuals ml^{-1} in 150 to 500 L tanks with a low water flow of filtered and UV-treated seawater (Heasman and Savva, 2007). The tank is aerated from the base to ensure high dissolved oxygen levels and that the larvae are evenly dispersed throughout the tank (Heasman and Savva, 2007). Also dark conditions have been observed to help that larvae spread more evenly throughout the water column so that lights are mostly switched off in the hatchery (L. J. McPherson, JTA, pers. comm., March 2015). The abalone reach settlement stage after 6 to 8 days at the recommended rearing temperature of 16 to 18 °C (Heasman and Savva, 2007; Table 1.1). Competent larvae are then transferred to nursery tanks.

In the nursery, the abalone are reared on submerged plastic plates that are colonised with a diatom-dominated biofilm. The algae attract the larvae to settle and provide enough food for the larvae to metamorphose (Heasman and Savva, 2007). The nursery tanks are constantly supplied by filtered seawater at the local environmental temperature of usually 16 to 24 °C and a high level of aeration is provided to ensure high oxygen levels (Heasman and Savva, 2007; A. Krsinich, Jade Tiger Abalone (JTA), pers. comm., November 2013; Table 1.1; Fig. 1.1). Post-larval growth is dependent on temperature and the abalone reach a shell length (SL) of approximately 1.5 mm after 30 to 70 days at 20 to 24 °C and 14 to 16 °C, respectively (Heasman *et al*., 2004). The manual for rearing of *H. rubra* suggests transferring the abalone at this size of 1.5 mm from the plastic plates to raceways with diatom biofilms

and formulated feed powder (Heasman *et al*., 2004). Yet, the largest aquaculture farm in Australia, Jade Tiger Abalone (JTA), in Indented Head, Victoria, transfers their stock at a larger size of 10 to 15 mm SL (L. McPherson, JTA, pers. comm., December 2016). The abalone are gradually weaned onto the formulated diet only and remain in the raceways until harvest at approximately 80 to 90 mm SL (Table 1.1).

Various types of tanks are used for the grow-out phase throughout Australia (Heasman and Savva, 2007). The grow-out tanks used at JTA are described here because the animals used in this thesis were sourced from this farm (Table 1.1). In brief, the raceways are 20 m long x 1.8 m wide concrete slab tanks with a maximal water depth of 5 cm. Water is sourced from the nearby Port Phillip Bay and runs directly (open-flow) through the raceways at a flow rate of ~ 180 L min^{-1}. The water-flow direction is reversed every two weeks to prevent crowding of abalone because abalone move against the water flow and crowd at the inlet (A. Krsinich, JTA, pers. comm., November 2013). The oxygen level of the incoming water is usually fully saturated, yet oxygen commonly decreases to approximately 70% air saturation (O_2sat) at the end of the slab tank (Wassnig *et al.*, 2009; Wassnig *et al.*, 2010; Fig. 1.2). Water temperatures vary with the local environmental conditions between a minimum of 9 °C in winter and a maximum of 24 °C in summer (A. Krsinich, JTA, pers. comm., November 2013; Fig. 1.1). At JTA, the raceways are housed in rooms that are dark for 24 h because aquaculture farmers report less movement of abalone in darkness (A. Krsinich, JTA, pers. comm., November 2013).

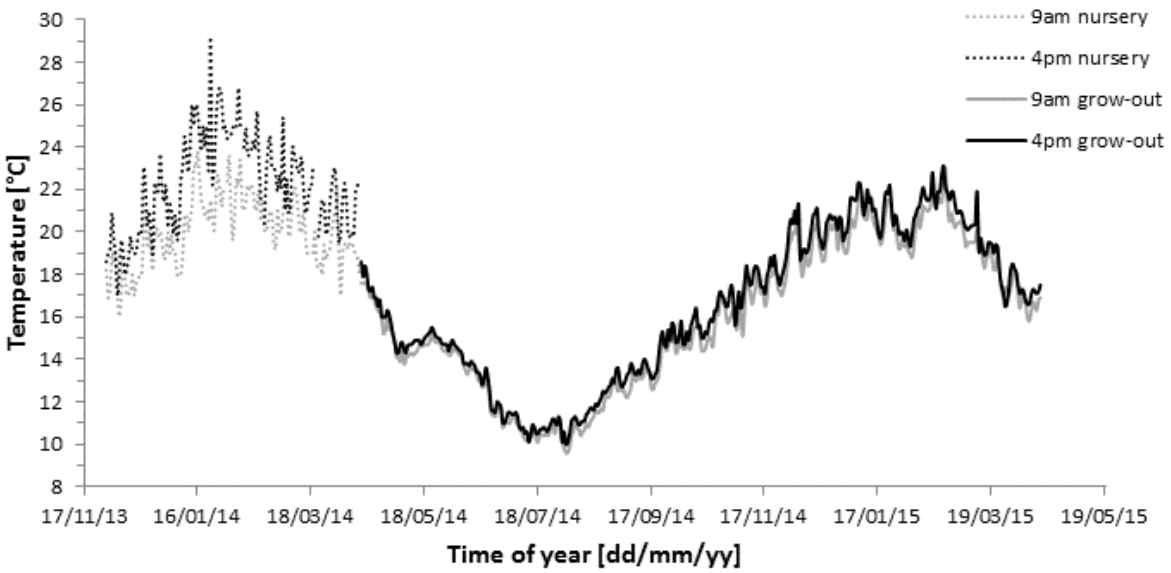

Fig. 1.1: Water temperatures in the nursery (dotted lines) and grow-out tanks (solid lines) at Jade Tiger Abalone (JTA). Data were collected at 9 am (grey lines) and 4 pm (black lines) from November 2013 to April 2015 (time when experiments with individuals from the nursery and grow-out tanks were performed). Data provided by JTA.

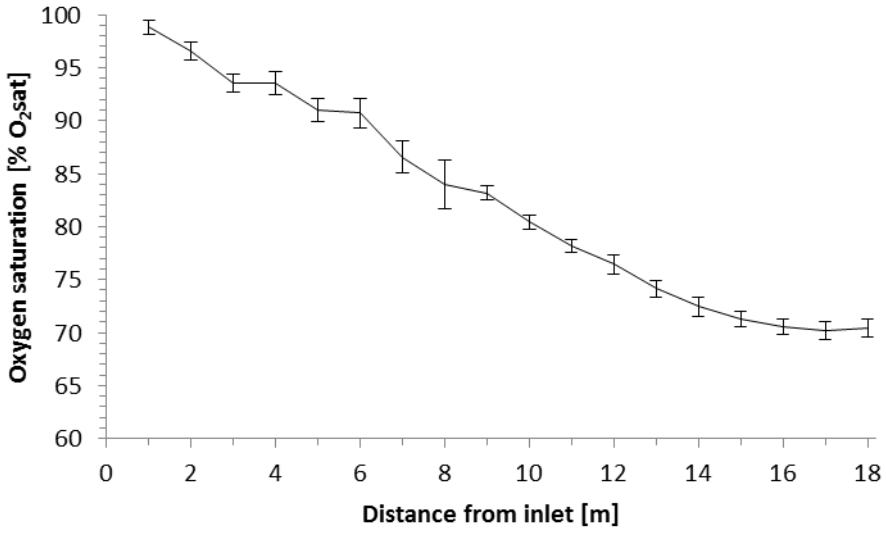

Fig. 1.2: Seawater oxygen saturation [% O_2sat] within the grow-out tank [m] at Jade Tiger Abalone. Oxygen saturation was measured using a hand-held oxygen probe (Oxyguard) at 3-h intervals on 28th and 29th October 2015. Temperature did not vary either during the measurement period or along the grow-out tank and was 17.9 ± 0.1 °C; mean ± SE; n = 7.

Aquaculture stock

While the established protocols result in a high yield, aquaculture farmers need to further maximise the growth potential and survival of abalone to meet the aforementioned expected production increase. Both, *H. rubra* and *H. laevigata* were the predominant commercial species at the beginning of Australian aquaculture. Yet, an interspecies hybrid from *H. rubra* females and *H. laevigata* males has been produced since 2007 in selective breeding programs and is becoming a key commercial asset (Li, 2008; Guo, 2009; Hamilton *et al*., 2009). The hybrid is produced commercially because it has higher growth rates, lower mortality rates during live transport, and higher meat yields than the parental pure species (Guo, 2009; P. Kube, CSIRO, pers. comm., November 2016; Fig. 1.3).

The superior traits and/or fitness are known as heterosis or hybrid vigour and have been demonstrated for several abalone hybrids worldwide (reviewed by Lafarga-De la Cruz and Gallardo-Escárate (2011)). Most research with the Australian hybrid, however, has addressed commercial needs such as meat quality and immune responses (Hooper *et al*., 2007; Hooper *et al.*, 2011; Hooper *et al.*, 2014; Mateos *et al*., 2010; Mateos *et al*., 2012; Dang *et al*., 2011; Dang *et al*., 2013). As such, little is known about how hybrid physiological and behavioural responses to environmental conditions differ compared to the parental species. A better understanding of hybrid performance under a variety of environmental and production conditions could help to further optimise abalone production, and explain why hybrids have a superior growth (Fig. 1.3).

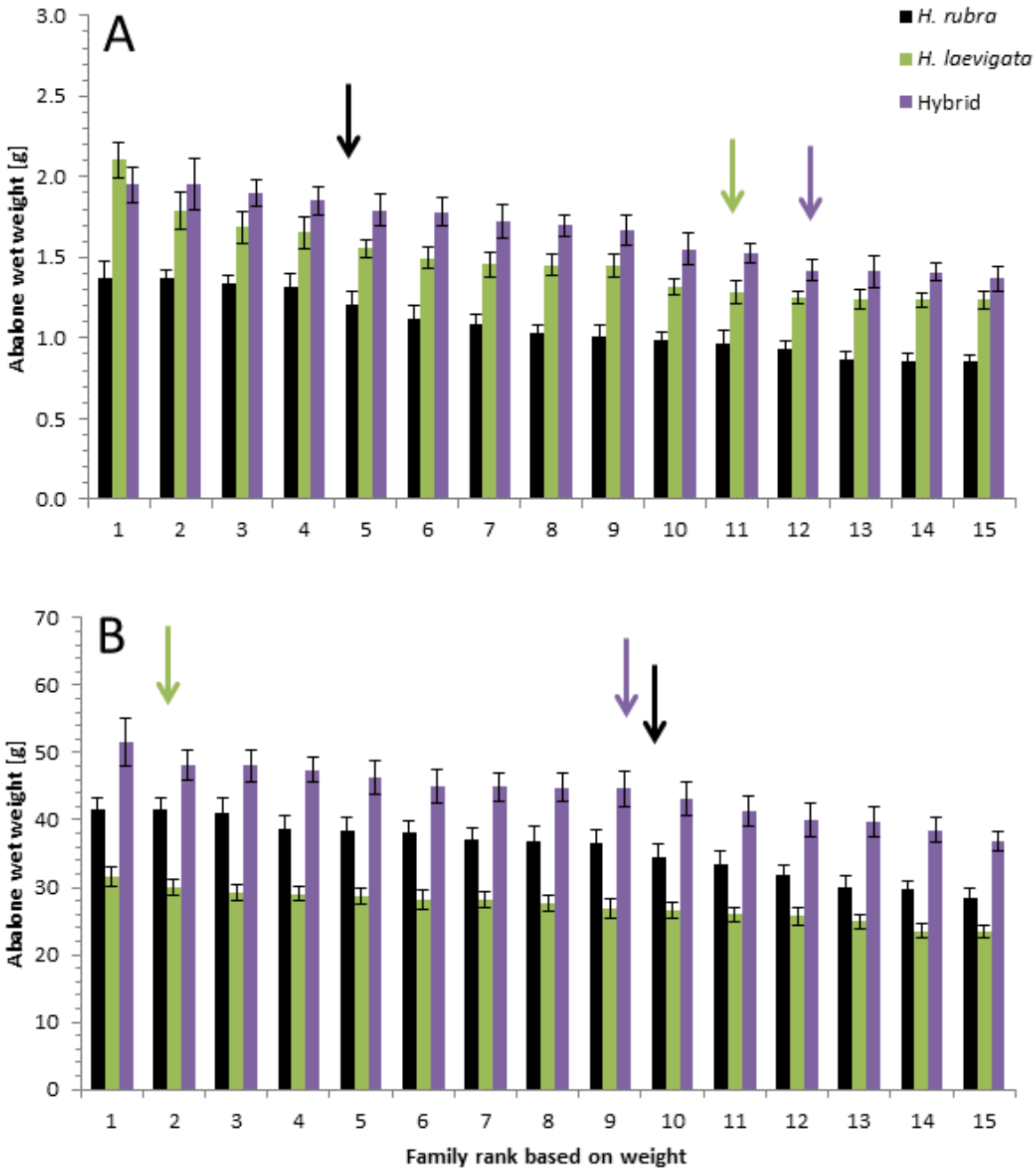

Fig. 1.3: Whole animal wet weights [g] of 9 months old (A) and 20 months old (B) *H. rubra* (black bars), *H. laevigata* (green bars), and their interspecies hybrid (purple bars). The abalone originate from different families from the 2013 year class and are ranked according to their weight. Vertical arrows indicate families that were used for chapters 5 and 6. Similar coloured vertical arrows in A and B indicate the same family. Data provided by Jade Tiger Abalone; mean ± SE; n = 15 to 39.

Responses to temperature and oxygen level

Physiology

Temperature and oxygen are major abiotic factors controlling physiological function in ectothermic animals, such as abalone (Somero, 2002). Physiological reaction rates change with temperature, and oxygen is required in the aerobic pathways that produce the energy needed to drive these reactions (Dahlhoff and Somero, 1993; Grieshaber *et al*., 1993). Animals in the wild often congregate in areas where the environmental conditions are optimal for physiological functions, and hence, promote optimal development, growth and reproduction (Fraenkel and Gunn, 1961; Dahlhoff and Somero, 1993; Tomanek and Somero, 2002; Morley *et al*., 2009). On aquaculture farms, however, abalone cannot escape culture environments and thus may be exposed to unfavourable growth conditions. In general, animals are able to survive outside their optimal environmental conditions, but physiological functions including metabolism can be impaired, which can slow development and growth (Jobling, 1981; Park *et al*., 2009; Romo *et al*., 2010).

Temperatures increase and oxygen levels decrease on aquaculture farms during summer months which may result in stressful conditions for abalone (Fig. 1.1 and 1.2). Indeed, abalone farmers often notice higher mortality rates of their stock during this time of year (Vandepeer, 2006). High temperatures are accompanied by decreased oxygen levels due to the lower solubility of oxygen at higher temperatures (Hindrum *et al*., 1996). Hence, it may be a combination of temperature and oxygen levels, amongst other suboptimal farm conditions, which result in stressful conditions and higher mortality for the abalone. Not all abalone types are equally affected by summer conditions on Australian aquaculture farms. Hybrid abalone have the lowest summer mortality rates, which may be indicative of a higher tolerance to increased temperatures and decreased oxygen levels in comparison to their pure parental species. Indeed, an increased thermotolerance in hybrids in comparison to pure parental species has been reported for several hybrids, including *H. discus hannai* Reeve × *H. kamtschatkana* Jonas, *H. rufescens* Swainson × *H. discus hannai*, and *H. laevigata* × *H. scalaris* Leach (reviewed by Lafarga-De la Cruz and Gallardo-Escárate (2011)). No such data exist for the *H. rubra* × *H. laevigata* hybrid and studies about tolerance to low oxygen conditions between pure and hybrid abalone have not been carried out.

Aerobic energy (ATP) production (oxidative phosphorylation) is 15- to 18-fold more efficient than anaerobic (glycolytic) energy production and thus the bulk of physiological processes are generally fuelled using aerobic pathways. Abalone are oxygen regulators and maintain

their oxygenanae consumption rate ($\dot{M}O_2$) over a range of oxygen tensions until a critically low oxygen level (P_{crit}) is reached (Jan and Chang, 1983). Oxygen consumption rate drops rapidly below the P_{crit} and individuals increasingly rely on less-energy-efficient anaerobic metabolism (Grieshaber *et al*., 1993; Pörtner and Grieshaber, 1993). Critical oxygen levels may be life-stage specific, as demonstrated for other invertebrates (Alter *et al.*, 2015). For abalone, however, changes in P_{crit} across development have rarely been studied. The P_{crit} of the European abalone *H. tuberculata* Linnaeus decreased with size, indicating a greater hypoxia tolerance in larger animals. The $\dot{M}O_2$ of small *H. tuberculata* juveniles with a dry weight (DW) of 0.02 g is more highly dependent on environmental oxygen in comparison to larger juveniles with 1.51 g DW (Gaty and Wilson, 1986). No such information across sizes is available for the Australian species *H. laevigata* and *H. rubra* or their interspecies hybrid. It has been reported, however, that long-term exposure of 10 g wet weight (WW) *H. laevigata* juveniles to oxygen levels below their P_{crit} of 81% O_2sat resulted in lower growth rates and decreased food consumption (Harris *et al*., 1999). Oxygen levels are commonly around 70% O_2sat at the end of slab tanks at the JTA aquaculture farm (Wassnig *et al.*, 2009; Wassnig *et al.*, 2010; Fig. 1.3). This suggests that *H. laevigata*, and perhaps *H. rubra* and their interspecies hybrid, may be limited in their scope for growth due to suboptimal oxygen levels in commercial grow-out tanks. Determining P_{crit} of *H. rubra, H. laevigata,* and their interspecies hybrid may help to understand hybrid vigour. For example, a lower P_{crit} in hybrids could translate to a higher scope for growth in a variable oxygen environment and explain interspecies differences in growth rate.

In ectothermic species, such as abalone, physiological reaction rates are dependent on temperature. Oxygen consumption rate correlates typically positively with temperature throughout the ecological range of most species (Dahlhoff and Somero, 1993; Grieshaber *et al*., 1993). This correlation, however, is finite and $\dot{M}O_2$ will reach a maximum at a critical temperature above which animals can no longer function and $\dot{M}O_2$ will drop abruptly as animals approach death (Dahlhoff and Somero, 1993). Traditionally, the critical upper temperature (CT_{max}) is determined by exposing an animal to gradually increasing temperatures and measuring the temperature at which an animal loses balance and mobility (Cowles and Bogert, 1944). This method provides limited additional information on the current physiological knowledge regarding improved performance of hybrids. Instead, knowledge about temperatures at which $\dot{M}O_2$ drops significantly may be beneficial to understand survival and growth rates during thermal stress in abalone.

Optimal and critical temperatures are species and life stage/age specific, but little is known about how they change during the lifecycle of *H. rubra*, *H. laevigata,* and their interspecies hybrid (Leighton, 1972; Sawatpeera *et al*., 2001; Steinarsson and Imsland, 2003; Searle *et al*., 2006; Stone *et al*., 2013; Schaefer *et al*., 2013). Thermal preferences and CT_{max} in adult (~ 80 mm SL) *H. rubra* and *H. laevigata* are only slightly different between the two species. Thermal preference was 17 and 19 °C and CT_{max} was 27 and 28 °C for *H. rubra* and *H. laevigata*, respectively (Gilroy and Edwards, 1998). These values were determined via behavioural studies and it is yet unknown how $\dot{M}O_2$ responds to temperature, with the exception that $\dot{M}O_2$ is stable between 17 and 19 °C in both species (Harris *et al*., 2005). Understanding the relationship between temperature and $\dot{M}O_2$ in *H. rubra*, *H. laevigata*, and their interspecies hybrid at different sizes/ages will be advantageous to predict growth rates and better understand hybrid vigour. For example, $\dot{M}O_2$ in ectothermic species can change in response to selective breeding (Ksiazek *et al*., 2004). Controversy exists regarding whether $\dot{M}O_2$ is elevated or lowered in animals with improved growth (reviewed by Burton *et al*. (2011)). The "compensation hypothesis" presumes that higher growth in individuals is due to a lower resting $\dot{M}O_2$. The reduced energy costs for the maintenance of baseline metabolism can instead be channelled into growth. Conversely, the "increased intake hypothesis" suggests that individuals with higher growth rates possess a higher resting $\dot{M}O_2$ and an increased maximum $\dot{M}O_2$. This enables the individual to gain more energy per unit time and results in improved growth rates. It has been suggested that the latter hypothesis might be only valid for environments with an excess supply of food, which is the case in aquaculture facilities (reviewed by Burton *et al*. (2011)).

Understanding differences in other physiological parameters, such as enzyme activities or heart rates, might also be helpful to better understand hybrid vigour in abalone. Abalone frequently rely on anaerobic energy even in the presence of oxygen (Gäde, 1988; Baldwin *et al*., 1992). Given that anaerobic energy production is inefficient in comparison to aerobic energy production (Lee and Lee, 2011), a lower dependency on anaerobic energy production in hybrids, for example, could reveal an energetic advantage over pure species and ultimately translate into faster growth. Heart rate is generally positively correlated with $\dot{M}O_2$ and thus determination of heart rate can give valuable insight into the metabolism of an animal in cases where measuring $\dot{M}O_2$ is difficult (Marshall and McQuaid, 1992). Aquaculture farms are complex environments where multiple abiotic and biotic factors may synergistically affect the physiology of abalone. Establishing the correlation between heart rate and $\dot{M}O_2$ in

the laboratory is the first step to be able to collect heart rate data from sentinel abalone on the farm to better understand how the aquaculture environment influences energetics and growth. Enzyme activities and heart rate have been measured in this thesis on the two pure species and their interspecies hybrid. Determinations were conducted with selected life stages and a more detailed introduction to each parameter is given in respective chapters.

Behaviour

Determinations of movement patterns under different temperature and oxygen levels can also be useful because changes in movement might indicate suboptimal conditions for the individuals (Cenni *et al*., 2009; Cenni *et al*., 2010; Robinson *et al*., 2013). Alterations in behaviour influence weight gains due to energy expenses for locomotion, mucous production, and varying food intake (Cenni *et al*., 2009). Because behaviour can influence body weight it potentially influences growth rates and thus could help to understand hybrid vigour.

Abalone behaviour changes drastically across life-stages. The inactive embryos develop into constantly swimming trochophore and veliger larvae. During development, larvae become increasingly demersal and remain benthic after successful metamorphosis (Leighton, 1989). To date, no studies have quantified the swimming behaviour of abalone larvae, thus, it remains unknown if swimming behaviour changes during larval development, with varying environmental parameters, and/or type of abalone similar to other free-swimming larvae (Hidu and Haskin, 1978; Wendt, 2000). Furthermore, assessing larval swimming behaviour can assist in determining optimal rearing conditions which may be different for pure species and hybrids. Behavioural thermoregulation drives mobile species to congregate within a preferred range along a thermal gradient (Fraenkel and Gunn, 1961). This behaviour of larvae can optimise performance traits to enhance survival and fitness. For example, temperatures can be selected to optimise digestive efficiency which will enhance growth rates (Green and Fisher, 2004; Chapperon and Seuront, 2011a; Chapperon and Seuront, 2011b). Hence, determinations of preferred temperature ranges across larval development could assist in improving hatchery production.

Behavioural studies on juvenile abalone have gained more attention, especially with regards to feeding activity (Tutschulte and Connell, 1988; Day and Branch, 2002; Allen *et al.*, 2006; Buss *et al*., 2015; Currie *et al.*, 2016). As juveniles and adults, abalone are considered to be relatively sedentary and movement is assumed to occur mainly during disturbance or to find

food (Shepherd, 1973; Werner *et al*., 1995; Donovan *et al*., 1999; Cenni *et al*., 2009; Robinson *et al*., 2013). Yet, *H. rubra* and *H. laevigata* show distinct behavioural patterns. In the wild, juvenile and adult *H. rubra* move out nocturnally from their sheltered areas to search for food. Conversely, juvenile and adult *H. laevigata* are often found quite stationary on rocks where they can remain for several years (Shepherd, 1973). Aquaculture farmers have reported similar observations of *H. rubra* being more active when compared to *H. laevigata,* with their interspecies hybrid possessing an intermediate movement behaviour (A. Krsinich, JTA, pers. comm., November 2013). The differences in movement behaviour between both two pure species and their hybrid, however, have not been scientifically demonstrated. Yet, determining movement patterns and overall activity may reveal an energetic advantage of the hybrid over pure species and thus a higher scope for growth. It may be possible that hybridisation results in a change in behaviour that favours energy being directed to growth compared to the pure parental species. For example, a lower activity of hybrids may provide an energetic advantage due to lower energy expenses for locomoion. Indeed, progeny of *H. laevigata* that originated from breeding programs targeting higher growth rates travelled less than progeny originating from wild abalone (Robinson *et al*., 2013). Alternatively, also a higher activity of hybrids may provide an energetic advantage over pure parental species due to a higher foraging success. In line with this reasoning, it has been demonstrated that cultured hybrids have a higher food intake than *H. laevigata* (Currie *et al*., 2016).

Scope of the thesis

Aims and hypotheses

The aim of this comparative study was to increase the understanding of the improved growth (Fig. 1.3) of the *H. rubra* × *H. laevigata* hybrid, that is commercially produced at JTA, in comparison to its pure parental species. Physiological responses, in terms of $\dot{M}O_2$, P_{crit}, heart rate, and enzyme activities, and behavioural responses, in terms of larval swimming speed and behavioural thermoregulation, and juvenile movement activity, of hybrids, *H. rubra,* and *H. laevigata* were tested under a variety of environmental and production conditions commonly experienced by the abalone at the aquaculture farm JTA. All three types of abalone were studied from larval through to adult stages with an aim to identify species and life-stage differences. It was hypothesized that hybrids have higher growth rates when compared to parental species because the hybrid:

- is less active,
- is less sensitive to fluctuations in abiotic factors, and/or
- has a higher metabolic rate and thus gains more energy per time unit ("increased intake hypothesis").

Structure

In Chapter 2, the current knowledge regarding the physiology of all abalone species is reviewed. It summarizes the physiological responses of abalone to environmental and aquaculture farm stressors and identifies knowledge gaps that should be addressed to maintain and improve abalone aquaculture in the face of increased environmental variability. *Published as: Morash AJ, Alter K (2016) Effects of environmental and farm stress on abalone physiology: perspectives for abalone aquaculture in the face of global climate change. Reviews in Aquaculture 8: 342-368.*

In Chapter 3, it is investigated whether the current range of hatchery conditions affects the respiratory response of embryos, trochophore larvae, and veliger larvae of *H. rubra* × *H. laevigata* hybrids. Parameters measured include stocking density, oxygen levels of 100 to 0% O_2sat, and contrasting light conditions. *Published as: Alter K, Andrewartha SJ, Elliott NG (2016) Hatchery conditions do not negatively impact respiratory response of early life-stage development in Australian hybrid abalone. Journal of Shellfish Research 35: 585-591.*

In Chapter 4, Chapter 3 is complemented by exploring how ecologically relevant temperatures (12 to 25 °C) influence veliger swimming behaviour, thermal preference, and $\dot{M}O_2$ of *H. rubra*, *H. laevigata*, and their interspecies hybrid. *Published as: Alter K, Andrewartha SJ, Clark TD, Elliott NG (2017) Thermal preference increases during larval development of pure and hybrid abalone. Journal of Shellfish Research 36: 141-149.*

In Chapters 5 and 6, it is explored whether juvenile and adult hybrids have superior physiological and behavioural responses to long-term (2 to 3 weeks) elevated temperature and decreased oxygen (16 and 23 °C, 100 and 70% O_2sat) stressors compared to the pure parental species. In these chapters *H. rubra*, *H. laevigata*, and their interspecies hybrid are sourced from one family to minimize effects of genetic variation (Fig. 1.1). The physiological responses are determined at different levels of biological organisation ranging from enzyme assays to whole animal metabolism.

In Chapter 5, experiments are described in which juveniles are used to test whether the hybrid has a distinct behaviour and differs in its use of aerobic and anaerobic energy production in comparison to parental pure species which may contribute to its improved growth. Movement and $\dot{M}O_2$ are measured for two days at chronic exposure to acclimation conditions and subsequently during acute oxygen decrease to determine P_{crit}. Further, tauropine and lactate dehydrogenase activities are measured in muscle tissue samples to determine the anaerobic contribution to the metabolism of juveniles.

In Chapter 6, adults of *H. rubra*, *H. laevigata,* and their interspecies hybrid are exposed to increasing temperatures at their respective acclimation oxygen level. Oxygen consumption rate, heart rate, and behaviour are assessed at 2 °C intervals to determine upper critical temperatures for each biological parameter. *Published as: Alter K, Andrewartha SJ, Clark TD, Morash AJ, Hellicar AD, Leon R, Elliott NG (2017) Hybrid abalone are more robust to multi-stressor environments than pure parental species. Aquaculture 487: 25-34.*

In Chapter 7, the findings from the data chapters are brought together to determine physiological and behavioural advantages of the hybrid in comparison to pure species and to identify changes across life-stages. Finally, this chapter highlights the implications of this thesis on aquaculture practices and suggests future research directions.

CHAPTER 2: Physiology of cultured abalone

All of the research contained within this chapter has been published as:

Morash AJ, **Alter K** (2016) Effects of environmental and farm stress on abalone physiology: perspectives for abalone aquaculture in the face of global climate change. *Reviews in Aquaculture* **8:** 342–368.

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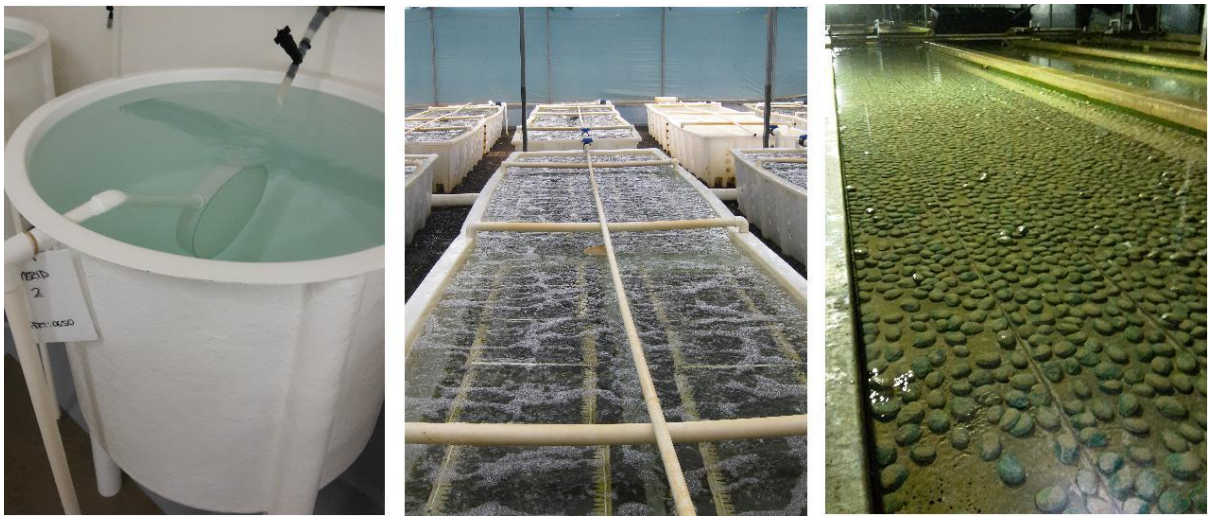

Set-up for rearing abalone in the hatchery (left), nursery (centre) and grow-out tanks (right) at the aquaculture farm "Jade Tiger Abalone" in Indented Head, Victoria, Australia, which kindly provided their abalone for on-farm and laboratory experiments.

Effects of environmental and farm stress on abalone physiology: Perspectives for abalone aquaculture in the face of global climate change

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Abstract

Many abalone farms are reliant on coastal water inputs which are subject to fluctuations in environmental variables such as temperature, oxygen, CO_2, and salinity. Near future climate change scenarios predict that there will be more frequent extreme weather events which can exacerbate these fluctuations and potentially be deleterious to farmed abalone where these variables remain largely uncontrolled. In this review we have taken an in depth examination of current literature on the effects of environmental stress on abalone physiology and metabolism and how this affects their health and growth. In conjunction, also reviewed the effects of farm specific stressors such as ammonia, stocking density, handling, nutrition, disease, and the synergistic effects of these and environmental stressors on abalone physiology are reviewed. We have identified currents gaps in our knowledge of this under-studied species and have made predictions on the effects of climate change on future abalone production with suggestions for future research. In summary, it is expected that abalone will show reduced growth rates as more energy is invested in combating stresses rather than growth. Furthermore, disease outbreaks may become more frequent with greater fluctuations in temperature and salinity, both of which have large scale effects on immunity. The current body of knowledge is mainly on whole animal effects of stresses, but we know very little of their mechanistic foundation. Research in this area as well as investments in infrastructure will be pivotal in identifying and implementing strategic interventions to maintain a sustainable abalone industry in Australia.

Keywords: abalone, physiology, climate change, metabolism, aquaculture, Australia

Introduction

This review examines the current knowledge of abalone physiology and metabolism within the broader context of aquaculture and climate change. We review the structure and function of the abalone cardio-respiratory system as well as the metabolic and energetic costs associated with their environment. The main focus of this review will centre on the physiological responses to important environmental and farming stressors to determine gaps in our knowledge and propose the future of abalone research to advance farming techniques to keep abalone farming sustainable in the face of climate change.

Abalone are a widespread group of marine gastropods, from the genus *Haliotis*, inhabiting both tropical and temperate waters around the world. Abalone have long been prised for their gastronomic value throughout the world, but particularly in Asian countries. Australia has also become a major contributor to the global market with farms in South Australia, Victoria, Western Australia and throughout Tasmania. Within Australia the main species being produced are greenlip (*H. laevigata*) and blacklip (*H. rubra*) abalone and their hybrid (for a full review on abalone hybrids see Lafarga-De la Cruz and Gallardo-Escárate (2011)). Basic farming techniques have become fairly well established and the recent focus has been on genetic improvement programmes centred on animal growth and farm efficiency (Elliott, 2000). There has now been considerable attention placed on establishing the physiological response of animals to their environment, particularly in the face of climate change (Pörtner and Farrell, 2008; Somero, 2010). The on-farm environment is a complex ecosystem where many abiotic factors remain largely uncontrolled (eg. salinity, temperature, oxygen, CO_2). Many farms are reliant on local coastal water inputs which change throughout the seasons and can change diurnally depending on weather and tidal conditions. In contrast, farms strictly monitor such things as nutrition, stocking density, water flow rates, aerial exposure, and handling time in order to minimise stressors. Often these stresses do not occur in isolation and recent work determining aerobic scope and optimal physiological conditions for animal health suggests that multiple stresses can reduce the overall coping range of ectothermic animals (Pörtner and Knust, 2007). This will be of particular importance in the dynamic and challenging farming environment. The growth of these animals is underpinned by their metabolism and energy budgets throughout their life cycle. Increases in stress divert energy away from growth and into protective mechanisms which can slow down the production. Understanding the effects of stress on abalone physiology will aid in the

development of farming protocols to minimise stress, increase productivity and ensure the highest quality, healthy abalone.

This review is a synthesis of the current research on environmental and farm variables that can affect the health, growth, and quality of farmed abalone. Particular focus will be on the cardiorespiratory and metabolic physiology given its large influence on growth and performance in these animals.

I. Abalone anatomy and physiology

Abalone are a unique species quite unlike other molluscs, therefore it is pertinent to first discuss their anatomy and basic physiology. Abalone evolved during the Cretaceous period (66 Ma) and retained many ancestral features (Lee and Vaequier, 1995). Their anatomy limits them to habitats with fast flowing highly oxygenated water where respiration remains relatively passive. Abalone have a very sedentary lifestyle, often only moving short distances at night to feed when drifting seaweed is limited, and clamping on the rocks to avoid predation or dislodgement by wave action. Given the limited mobility they have developed many anatomical and metabolic strategies to exploit their environment, some of which may appear inefficient but have proven highly successful.

Anatomy

Abalone have a flattened spiral shaped shell with a row of respiratory pores extending from the head region and crossing over a pair of bipectinate gills (Fig. 1). Lateral cilia movement within the gills function as their "ventilatory pump" by drawing water in around the head region and the first anterior pores of the shell. Abalone also rely heavily on water currents as a type of "ram ventilation" forcing water over the gills which is advantageous in their habitat (Voltzow, 1983; Tissot, 1992; Taylor and Ragg, 2005) and has been shown to be highly important in farming abalone (Hahn, 1989; Aviles and Shepherd, 1996; Freeman, 2001). The smooth flattened shell protects against drag in the water while a strong muscular foot is used for attachment to the substrate and to prevent dislodgement by strong currents or predators. The foot is surrounded by an epipodium with sensory tentacles that allow the abalone to detect its environment. The internal organs are arranged in a spiral under the shell around a strong adductor muscle which connects the shell to the foot (Yonge, 1947). The mantle, also attached to the adductor muscle, surrounds the organs and secretes new material for shell

growth. The mantle has a clear slit which opens up around the head and the large bipectinate gills beneath the respiratory pores (Voltzow, 1983). Water flows over the gills where it meets the anus at the posterior end, picks up waste, and leaves via the posterior pores (Harrison, 1962). The internal organ anatomy of the abalone is quite simple and includes a kidney, reproductive organ, and digestive gland, all of which are interconnected by a series of sinuses and blood vessels and a three chambered heart.

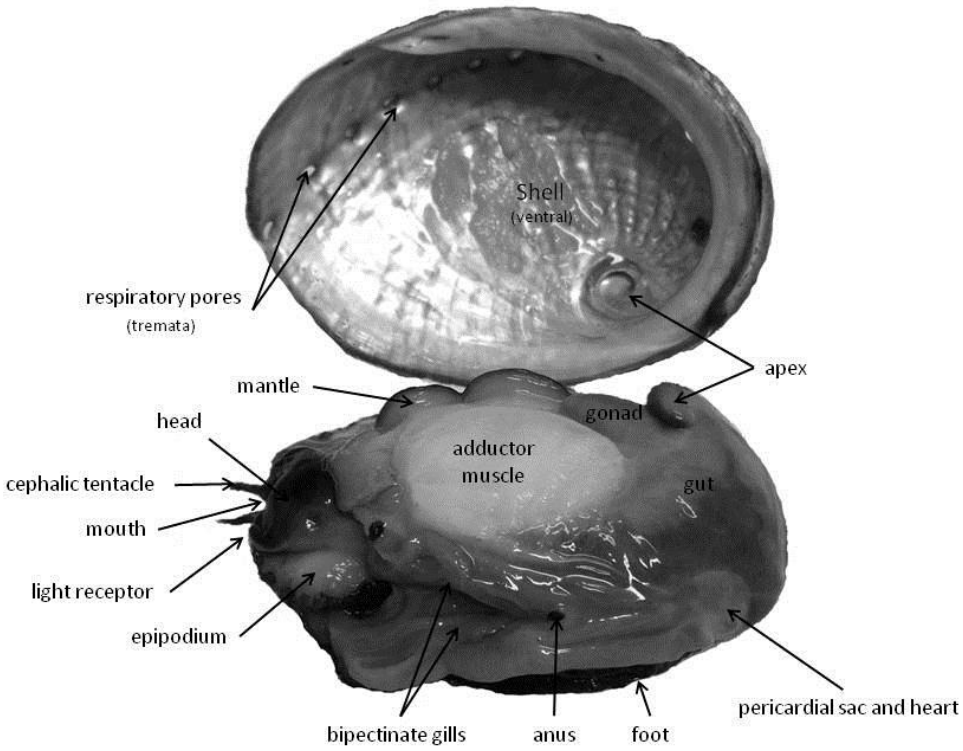

Fig. 2.1: Abalone anatomy. Ventral shell view and dorsal tissue view of major organs and muscle tissues.

Cardiorespiratory and Metabolic Physiology

Abalone have an open circulatory system consisting of a heart, arteries, veins, and various sinuses throughout the body (Jorgensen *et al.*, 1984). Although the system is open, there is still some degree of directionality and haemolymph can be shunted around the body to various organs under stressful conditions (see below). The haemolymph composes 55% of the weight of the abalone (shell excluded) which is consistent with the open circulatory design in which tissues are bathed in haemolymph. Not all organs, however, are bathed in equal amounts or at the same rate. For example, in *H. cracherodii* Leach the kidneys and epipodium have the largest volume of haemolymph although the kidney exchanges 1 volume per minute while the epipodium only exchanges 1/3 per minute. In contrast, the digestive gland has a very small volume but exchanges up to 4 volumes per minute while the adductor muscle constitutes 66% of the wet body tissue weight, but only receives 27% of the cardiac output (Jorgensen *et al.*, 1984). Only 3% of the total haemolymph volume is pumped per minute by a very small heart (0.04% body weight), thus there is limited exchanges of gases and waste when compared to animals with closed circulation. The low pressure of an open circulatory system combined with highly resistant vasculature throughout the gills restricts oxygenated haemolymph from returning to the heart to be pumped around the body. Consequently, the pericardial fluid volume remains at a constant level, therefore pumping of the ventricle creates a negative pressure in the auricles which draw in oxygenated haemolymph from the gills (Fretter and Graham, 1994). The gills of the abalone have been described as archaic and inefficient because they are reliant on external water movement even in highly oxygenated water. This is supported by the sedentary lifestyle of abalone and reliance on anaerobic metabolism (see below). A comprehensive study on gill perfusion and oxygen uptake in resting *H. iris* Gmelin, however, has shown that abalone respiration is roughly equivalent to other "competent" water breathers (Ragg and Taylor, 2006b).

Oxygen transport is extremely species-specific in abalone and this likely represents the diverse habitats and large latitudinal range over which they exist (tropical to temperate). The extraction efficiency of the abalone gill is only about 50%, of which 60 to 80% enters the haemolymph while the rest is used directly by the gills to support ciliary movement (Fretter and Graham, 1994). Abalone also have a low haemocyanin (Hcy) carrying capacity for oxygen (16 µg L^{-1}) and oxygen transport via Hcy can range from 34 to 97% depending on species (*H. iris* 34%; *H. ruber* [sic *H. rubra*] 86%; *H. laevigata* 97%; *H. roei* Gray 91%) while the remaining oxygen flows unbound as dissolved oxygen in the haemolymph (Ainslie,

1980b). The Hcy concentration also varies between species (*H. iris* 12.6 g L^{-1}; *H. roei* 3.6 to 15 g L^{-1}, *H. laevigata* 2.4 to 14.2 g L^{-1}; *H. ruber* [sic *H. rubra*] 1 to 9.9 g L^{-1} (Ainslie, 1980a)). Abalone are considered to be oxyregulators, meaning they maintain oxygen consumption (MO_2) over a range of environmental oxygen levels, until oxygen reaches a critical threshold at which oxygen demands can no longer be met in the animal (Jan and Chang, 1983). Abalone have developed several mechanisms to regulate or increase oxygen uptake, particularly during times of stress. For example, in response to high ammonia, *H. laevigata* can increase oxygen uptake by 1.88 fold (Harris *et al.*, 1998), while *H. kamtschatkana* can increase uptake by 2.3 to 3.5 fold during crawling (Donovan and Carefoot, 1997). Water flow over the gills is biassed towards the right gill under resting and unstressed conditions. During periods of activity or increased demand for oxygen, however, abalone can divert more haemolymph to the left gill for increased oxygen uptake (Ragg and Taylor, 2006a). Other marine gastropods such as limpets have the ability to use other body surfaces where the haemolymph comes close to the surface as sites for gas exchange (Kingston, 1968; Ruppert and Barnes, 1994). Abalone may also make use of other ventilatory sites such as the mantle (Voltzow, 1994) but it is unclear to what extent this mechanism is used, particularly during stress events. Furthermore, oxygen which remains bound to Hcy under resting conditions can be unloaded and used as a reserve during hypoxia (Wells *et al.*, 1998).

Abalone are facultative anaerobes and regularly rely on anaerobic metabolism even in the presence of oxygen. For example, the cost of transport for a crawling abalone has been estimated to be approximately 54% anaerobic in the foot muscle (Donovan *et al.*, 1999). Abalone produce d-lactate and opines (mainly tauropine) as anaerobic end-products depending on the metabolic state (Baldwin *et al.*, 1992). Tauropine is produced from taurine, (the most abundant amino acid in abalone) during "active" anaerobiosis, for example crawling, twisting or clamping resulting in tissue hypoxia as oxygen is depleted (Baldwin *et al.*, 1992). In contrast, environmental hypoxia induced anaerobiosis results in the production of lactate (Baldwin *et al.*, 1992). Despite the consistent production of acidic by-products, abalone have a limited pH buffering system in the haemolymph (Wells and Baldwin, 1995) which may lead to a metabolic acidosis during energetically costly movement or hypoxia. In fact, righting activity can lead to long recovery times, likely due to a combination of limited energy reserves and slow metabolic waste removal by the open circulation (Wells and Baldwin, 1995). This is substantiated by increased oxygen consumption lasting up to 5 h after exposure to strong currents causing the abalone to clamp down on the substrate (Donovan

and Taylor, 2008), and a 48 or 120 h recovery period after exposure to air in *H. iris* before lactate and tauropine levels returned to control values, respectively (Ryder *et al.*, 1994).

Abalone have a distinct set of cardiorespiratory and metabolic characteristics that have evolved in a relatively stable environment. The next two sections describe the extent to which abalone can respond to major environmental and farming disturbances and how this impacts their growth through effects on metabolism.

II. Physiological response to environment

Two of the most important variables that govern biological systems are temperature and oxygen. Temperature dictates the rate at which biological reactions can occur and therefore underpins major physiological processes. Aerobic metabolism provides the majority of energy to fuel these processes, thus creating a requirement for oxygen. Two other major environmental challenges in the marine environment are salinity and CO_2/pH. Marine animals, particularly those living in shallow coastal areas where freshwater influx is high, may experience diurnal or seasonal salinity fluctuations that impair the chemical balance between the environment and the body fluids. This leads to active pumping of ions to regain homeostatic balance; an energetically costly process. Furthermore, many marine animals are now faced with challenging levels of CO_2 in the water causing changes in pH. Acid/base balance is an important factor in oxygen transport and waste removal and plays a major role in ion movement across the gills. It is also especially important for marine calcifiers whose shell growth is affected by the effects of pH on aragonite saturation. Together, these factors contribute to the energy requirements of the animals which can change based on any given environment. While most animals are living at or near their optimum environmental conditions, they can deviate from their optima within certain limits and still survive. This, however, is at the expense of many critical life history traits such as development, growth, and reproduction. Temperature, oxygen, salinity and pH/CO_2 are all critical to abalone physiology and often vary in intensive aquaculture systems relying on coastal water. In this section, the effects of these four critical environmental variables on abalone physiology are discussed with an emphasis on how these variables may impact abalone aquaculture in the future.

IIa. Temperature

In ectothermic animals, temperature is the main variable that controls the rate of most metabolic processes. There is a positive correlation between oxygen consumption (MO_2) and environmental temperature, but this is a finite relationship extending only between the critical thermal maximum (CT_{max}) and minimum (CT_{min}), above or below which animals can no longer function. In the wild, most animals congregate within a preferred temperature range (T_{pref}) along a thermal gradient (Fraenkel and Gunn, 1961) where oxygen delivery is maximal (Frederich and Pörtner, 2000). The T_{pref} represent best conditions for the most efficient metabolism, reproduction and growth (Dahlhoff and Somero, 1993; Tomanek and Somero, 2002; Morley *et al.*, 2009; Table 2.1). Within this range the MO_2 is maintained both at the whole animal level and at the level of the mitochondria. *H. laevigata, H. rubra,* and *H. corrugata* Wood maintain a constant MO_2 within their T_{pref} (Harris *et al.*, 2005; Romo *et al.*, 2010; Table 2.1) and are often found at their T_{pref} in their natural habitat (Dahlhoff and Somero, 1993). This temperature also corresponds to the highest growth rate in most species (Britz *et al.*, 1997; Harris *et al.*, 2005; Green *et al.*, 2011; Cho and Kim, 2012).

Outside their optimum range individuals adjust basic physiological functions to maintain basal metabolic demands (Jobling, 1981; Park *et al.*, 2009; Romo *et al.*, 2010). As temperature increases, the rate of physiological reactions increase which requires more energy and thus more oxygen. Few studies have been conducted that investigate metabolic rate in response to temperature but two examples indicate that $\dot{M}O_2$ is lower in *H. tuberculata* when exposed to 8 ºC in comparison with 16 or 24 ºC (Gaty and Wilson, 1986) and aerobic metabolism is restricted above 20 ºC in *H. iris*, due to a limited capacity for oxygen storage in haemolymph (Wells *et al.*, 1998). In general, investigations on abalone critical thermal minima (CT_{min}) are rare (Leighton, 1972; Chen and Chen, 1999; Takami *et al.*, 2008) and critical thermal maxima (CT_{max}) were almost exclusively determined by measuring the temperature at which abalone lose detachment from a vertical surface (modified according to Jobling (1981)). Furthermore T_{pref} was measured either by behavioural thermoregulation or growth rates (Table 2.1). There is little information available in which the optimum and critical temperatures have been determined through metabolic rate. Measuring thermal preferences and critical thermal maxima using metabolic rate will provide a more accurate and objective representation of these values and enable predictions of growth rates during thermal stress.

Table 2.1: Critical thermal maxima (CT_{max}) and thermal optima for growth (T_{pref}) for various species of *Haliotis*. Notice the high CT_{max} for *H. fulgens*, *H. corrugata*, and *H. diversicolor supertexta*. Thermal responses were almost exclusively determined via behavioural thermoregulation or growth rates (except Wang *et al*. (2006)).

Species (size)	CT_{max} (°C)	T_{pref} (°C)	Reference
Adult			
H. cracherodii (100 to 150 mm)	26-27		Hines *et al*. (1980)
H. laevigata (84 mm)	28	19	Gilroy and Edwards (1998)
H. rubra (79 mm)	27	17	Gilroy and Edwards (1998)
H. rubra (94 mm)	-	15-17	Wang *et al*. (2006)
Juvenile			
H. corrugata (25.7 mm)	32	25	Díaz *et al*. (2006)
H. corrugata (12.2 to 26.4 mm)	-	21	Leighton (1974)
H. discus hannai (20 mm)	-	>17	Hoshikawa *et al*. (1998)
H. discus hannai (40 mm)	-	20	Cho and Kim (2012)
H. diversicolor supertexta (unspecified)	34-37	-	Chen and Chen (1999)
H. fulgens (28.7 to 30.5 mm)	34	25	Díaz *et al*. (2006)
H. fulgens (30.5 to 38.9 mm)	-	24-27	Leighton (1974)
H. iris (10 to 30 mm)	28-29	17-22	Searle *et al*. (2006)
H. kamtschatkana (45 to 55 g)	24-27	0	Paul and Paul (1998)
H. kamtschatkana (20 mm)	-	15	Hoshikawa *et al*. (1998)
H. midae (30 to 45 mm)	28	24	Hecht (1994)
H. midae (17.5 mm)	-	12-20	Britz *et al*. (1997)
H. rubra (32 mm)	-	17	Harris *et al*. (2005)
H. rubra (unspecified)	27-28	-	Drew *et al*. (2001)
H. rufescens (46 to 59 mm)	28	19	Díaz *et al*. (2000)
H. rufescens (9.9 to 20 mm)	-	18	Leighton (1974)
Larvae			
H. corrugate	-	18-21	Leighton (1974)
H. fulgens	-	20-23	Leighton (1974)
H. rufescens	-	15-18	Leighton (1974)
H. sorenseni	-	16-18	Leighton (1972)
Hybrids			
H. rufescens X H. discus hannai (34 mm)	28	-	Lafarga-De la Cruz *et al*. (2013)
H. discus hannai X H. discus hannai (25 mm)	28	-	Cheng *et al*. (2006)

Beyond the optimum range, critical high temperatures will start to negatively affect biological structures and processes eventually leading to death. Exposure to temperatures outside of T_{pref} may affect abalone populations and communities by causing varying development times (Grubert and Ritar, 2004; Rogers-Bennett *et al.*, 2010), stunted or slower growth (Onitsuka *et al.*, 2008; Byrne *et al.*, 2011), altered susceptibilities to diseases (Raimondi *et al.*, 2002; Rosenblum *et al.*, 2005; Vilchis *et al.*, 2005), and mortalities (Leighton, 1974; Raimondi *et al.*, 2002; Searle *et al.*, 2006). These changes are mediated mainly by temperature influences on metabolic rate (Wells *et al.*, 1998; Hickey and Wells, 2003; Vosloo *et al.*, 2013c), but also through temperature sensitive feed conversion ratios (Britz and Hecht, 1997; Lopez *et al.*, 1998; Sales and Britz, 2001; García-Esquivel *et al.*, 2007). The size and developmental state of the individual can also influence its thermal tolerance (Leighton, 1972; Sawatpeera *et al.*, 2001; Steinarsson and Imsland, 2003; Searle *et al.*, 2006; Stone *et al.*, 2013; Schaefer *et al.*, 2013). *H. rufescens* T_{pref} increased across development until 30 mm SL and then declined with further increasing SL (Steinarsson and Imsland, 2003). *H. iris* also has a higher T_{pref} at SL of 10 and 30 mm (21 ºC) compared with 60 mm individuals (17 to 18 ºC; Searle *et al.*, 2006). It is critical to have a robust and accurate understanding of the effects of temperature on abalone metabolism and growth across all life stages to be able to predict the future effects of rising ocean temperatures on abalone farming.

Thermal stress initiates a suite of physiological responses that are initially centred on increasing energy production through alternate pathways. Abalone are facultative anaerobes and frequently rely on anaerobic metabolism for energy. Yet, anaerobic metabolism is a short term mechanism for which to supplement energy and above certain temperatures will also be limited. Tauropine and lactate dehydrogenases (TDH and LDH, respectively) catalyse the reduction of pyruvate to tauropine and D-lactate, respectively (Gäde, 1988; Baldwin *et al.*, 1992; O'Omolo *et al.*, 2003). In *H. iris*, TDH activity declines at temperatures above 20ºC (Hickey and Wells, 2003) which correlates well with maximum habitat temperatures and experimentally determined T_{pref} of the species (18 °C) (Searle *et al.*, 2006). Thermal stress can also induce a switch in metabolic fuels from carbohydrates and fats to protein oxidation. The ratio of $\dot{M}O_2$ to nitrogen excretion rate (O/N) gives insight into the physiological state of the individual based on the metabolic fuel preference (Somero, 2002). Protein based catabolism as indicated by higher nitrogen excretion is often associated with stress (Bayne, 1973). For example, upper thermal limits in *H. midae* Linnaeus were revealed by

demonstrating that protein utilization increased during exposure to 19 ºC compared with lower temperatures (Vosloo *et al.*, 2013c). Information about the metabolic state allows estimations about energy cost and hence, calculations of temperature dependent growth, which has been demonstrated frequently over long experiment times by manually measuring growth (Leighton, 1974; Britz *et al.*, 1997; Searle *et al.*, 2006). Correlations between catabolic substrates and net growth rates have been determined in other molluscs (oysters or blue mussels; Tedengren and Kautsky, 1986; Shpigel *et al.*, 1992), but only recently in greenlip abalone, *H. laevigata* (Stone *et al.*, 2013) over a range of temperatures.

When changes in substrate oxidation cannot fully compensate for the increased metabolic demand at high temperatures animals may begin to show signs of oxidative stress. This will result in the onset of repair and degradation processes to protect the animal from further damage (Sokolova and Pörtner, 2003). Thermal stress can increase the formation of reactive oxygen species (ROS) beyond put.ative levels, which can lead to damage of almost all biological macromolecules (Halliwell and Gutteridge, 1985). The effects of ROS can be mitigated by antioxidant defence mechanisms or through heat-shock proteins (HSPs), both of which have been investigated in abalone. Antioxidant enzymes degrade ROS into less reactive compounds (Hermes-Lima *et al.*, 1998), while HSPs refold or degrade damaged proteins (Panaretou and Zhai, 2008). De Zoysa *et al.* (2009) investigated a suite of antioxidant enzymes in *H. discus discus* Reeve and found that abalone have a well developed antioxidant defence system for thermal stress (28 ºC). Individuals up-regulated mRNA expression of catalase (CAT), thioredoxin peroxidase (TPx) and glutathione peroxidase (GPx) while thioredoxin-2 (TRx-2) expression remained unchanged after 24 h-exposure. mRNA expression of two types of superoxide dismutases (SOD), magnesium SOD (MnSOD) and copper/zinc SOD (CuZnSOD), were variable and depend on the time scale that the animal was exposed to the stressor. Previous studies have shown similar results, in that SOD expression in abalone during heat exposure was highly versatile depending on both the analysed SOD isoform and tissue (Kim *et al.*, 2007). It is clear that antioxidant defence mechanisms exist in abalone but we lack a comprehensive outlook on their tissue specific function and the limits of their ability to scavenge ROS during thermal stress. If ROS production exceeds the limits of these antioxidant enzymes, damage will occur to cellular components. In this instance the next line of defence is the HSP response.

Similarly to the antioxidant enzymes, HSP expression has been shown to be highly variable in abalone. HSP levels change according to the time scale that the individual had been

exposed to the stress as well as the thermal history of the individual. Li *et al.* (2012) determined the HSP70 response of *H. discus hannai* to chronic and acute thermal exposure. After a 4 month-exposure individuals cultured at extreme temperatures (8 and 30 ºC) showed higher HSP levels that individuals held at 12 or 20 ºC. Maximum HSP expression was positively correlated with the previous acclimation temperature when exposed to an acute severe temperature (38 ºC) (Li *et al.*, 2012) indicating that the HSP response in abalone may be susceptible to "pre-conditioning"; a process whereby a previous exposure to a stress may protect the animal from subsequent more severe stress (Mosser and Bols, 1988). Vosloo and Vosloo (2010) showed that HSP70 expression of *H. midae* varied depending on the duration (24h vs. 1 month) and the temperature (16, 19, or 22 ºC). Abalone are generally considered to have low capability to acclimate to chronically altered conditions as well as to withstand acute thermal shock (Gilchrist, 1995; Gilroy and Edwards, 1998). Acclimation may only be possible to temperatures which they commonly experience within their biogeographical distribution (Dahlhoff and Somero, 1993). Average habitat temperatures for *H. midae* are between 13.5 and 18 ºC (Vosloo *et al.*, 2013c) which may explain the lower HSP70 expression after a 1 month exposure to 16 or 19 °C. The protective effects of HSP70 were also apparent in a comparative study between *H. discus hannai* Ino populations (Cheng *et al.*, 2006). At present there are no data available for antioxidant and HSP expression in Australian abalone. This area of research will help farmers to understand the thermal limits of their animals and to predict their ability to cope with heat stress in the future.

A full understanding of abalone thermal tolerance and T_{pref} from a metabolic perspective is lacking. Data sets of this nature will be imperative in the face of climate change and rising water temperature. Many farms are reliant on water influx from coastal areas which are subjected to natural temperature fluctuations and summer mortality is already a growing concern for many abalone farms. The average summer water temperatures are within the coping range for abalone, but bouts of extreme high temperature are becoming more common and are reaching critical thermal maxima, above which abalone cannot survive, yet the mechanisms of thermal stress causing mass mortalities remains elusive. Furthermore, the increase in disease prevalence with warming waters compounds the stress in the animals resulting in mass mortalities. At moderate temperature levels within the coping range of the animals, the growth limiting effects present an interesting and potentially economically lucrative area of research for the abalone aquaculture industry. Thermal preferences and optimum growth temperatures are species and age/size specific yet we still know relatively

little in this area. Research on the effects of temperature before and after live transport have also received little attention to date. Live transport is composed of multiple stressors, therefore eliminating the effects of thermal stress pre and post shipment would aid in recovery and prevent mortalities.

IIb. Hypoxia

Dissolved oxygen is critical for animal growth. Fluctuations in water oxygen concentration occur naturally, particularly in coastal regions where tidal flow, decaying algal or phytoplankton blooms (Elston, 1983) and temperature fluctuations occur. Periods of hypoxia may also occur in land based aquaculture systems reliant on coastal water input, or as result of stocking density, water flow rate, aeration, exposure to air, biological oxygen demand (decaying waste or uneaten food) or changes in temperature (Hindrum *et al.*, 1996). Moreover, functional hypoxia may exist during periods of activity such as crawling, shell adhesion, or righting after being dislodged. These intense bouts of exercise often deplete the energetic reserves and increase oxygen demand beyond the rate of uptake (Baldwin *et al.*, 1992; Donovan *et al.*, 1999). The ability of abalone to withstand these periods of hypoxia is dependent on the extent/duration of the exposure and developmental stage of the animal.

A common tactic among cultured or lab abalone species in the face of low oxygen is to escape the tanks in search of more highly oxygenated water (Jan and Chang, 1983; Harris *et al.*, 1999). In cases where abalone cannot find well oxygenated water other adaptive responses must be employed to ensure survival. In the first instance, there are a series of physiological modifications which can occur to increase the uptake and transport of oxygen to the tissues. If the oxygen supply to the tissues remains below the demand, modifications at the cellular level will attempt to reduce oxygen demand, become more metabolically efficient, or employ mechanisms to protect against the damaging effects of hypoxia on cellular components.

The first line of physiological mechanisms that allow abalone to cope with fluctuating oxygen levels have been fairly well characterised (Ragg and Taylor, 2006a; Ragg and Taylor, 2006b). Oxygen uptake is mainly through the gills and is enhanced by movement of cilia on the gill lamellae, increased water flow over the shell and increased branchial blood flow. In a study separating the various tissue of an abalone, it was found that up to 14% of oxygen uptake can occur through the foot/epipodium (Taylor and Ragg, 2005). Under normoxic

conditions, oxygen uptake is primarily through the right gill, while the left gill is only recruited during periods of hypoxia to enhance surface area and oxygen uptake (Ragg and Taylor, 2006a). Despite the ability to increase uptake at the gill, the abalone circulation is geared more towards storage than delivery to the tissues (Wells *et al.*, 1998). Marine gastropod Hcy exhibits a reverse Bohr effect in which oxygen binds more tightly at low pH or high CO_2 partial pressure (Wells *et al.*, 1998). It is hypothesised that the reverse Bohr effect evolved in gastropods such as abalone to maintain oxygen levels during retraction into the shell and clamping to the surface (Wells *et al.*, 1998). In this energetically costly state, the pedal musculature becomes anaerobic and produces lactate and tauropine causing a metabolic acidosis of the haemolymph. The low pH increases the affinity of Hcy for oxygen allowing the oxygen to be conserved for longer periods and avoiding total anoxia during a hypoxic event (Wells *et al.*, 1998). At rest pH of the pedal sinus is 7.39 for *H. iris* and 7.35 for *H. rubra* (James and Olley, 1970; Wells *et al.*, 1998). pH of the pedal sinus can drop as low as 6.51 with expercise (*H. iris*) (Wells *et al.*, 1998), and 6.75 after exposure to air (*H. rubra*) (James and Olley, 1970). In some instances blood flow can be completely shunted away from the muscle in order to preserve oxygen for more oxygen-dependent tissue such as the kidney, digestive gland and gonad (Jorgensen *et al.*, 1984; Russell and Evans, 1989). Abalone appear to display an inter- and intraspecific variability in oxygen-carrying capacity (Ainslie, 1980b; Boyd and Bourne, 1995), implying adaptive responses to environmental or functional hypoxia (Wells *et al.*, 1998).

Haemolymph acid base balance is also disturbed during hypoxia. Cheng *et al.* (2004) reported a drop of 72% in pH and 58% in bicarbonate in abalone exposed to hypoxic water (2.11 mg L^{-1}) for 24 h. This drop in pH is likely associated with the increase in anaerobic metabolism and production of lactate. Abalone are highly reliant on anaerobic metabolism to provide partial compensation for energy production during hypoxia. Both lactate and tauropine have been used as stress indicators in abalone (Baldwin *et al.*, 1992). Phosphoarginine (PA) also provides a critical phosphate reserve for ATP synthesis during prolonged hypoxia (Shoubridge and Radda, 1984). The concentration of PA declines in red abalone (*H. rufescens*) exposed to hypoxia or air (Tjeerdema *et al.*, 1991), however, PA reserves are low in *H. lamellosa* Lamarck musculature (Gäde, 1988) thereby limiting their use during chronic hypoxia.

If the above mechanisms fail to meet the ATP demands, cells will shift to protective measures against the damaging effects of ROS produced during prolonged hypoxia. Similarly to

exposure to high temperature, abalone display a diverse range of antioxidant defences to hypoxia, and these responses can vary depending on the developmental age. Juveniles appear to have a considerable advantage over adults in terms of protection. Both GPx and SOD activity in *H. midae* are significantly higher in juveniles (40 mm; 12 g) than adults (60 mm; 50 g) during hypoxia (Vosloo *et al.*, 2013b). Adults also showed an increase in DNA damage during hypoxia which was not evident in the juveniles (Vosloo *et al.*, 2013a). GPx mRNA expression is also significantly higher in *H. discus discus* adults (50 to 60 g) during hypoxia, but was not compared to juveniles in this research (De Zoysa *et al.*, 2009). These differences are likely due to the variation in the environmental conditions of juveniles and adults. Adult abalone tend to be in relatively stable conditions whereas post-larval juveniles settling on diatom biofilms in intertidal areas may experience hypoxia during dark phase photosynthesis (Daume, 2006; Roberts *et al.*, 2007). Their limited size and mobility prevents them from escaping any diurnal fluctuation in hypoxia that may exist in their environment. Juvenile oxygen consumption rates are higher than adults under both normoxic and hypoxic conditions (Vosloo *et al.*, 2013c) and given the advantage in ROS protection may be able to survive and grow in unfavourable conditions in which adults cannot. It is currently unclear how long this advantage may persist, and why it is lost in adult abalone.

Decreased dissolved oxygen (DO) often results in poor growth. Moderate oxygen saturation (73%) significantly depresses specific growth rate whether measured by length or whole body weight in greenlip abalone (Harris *et al.*, 1999). This level of oxygen saturation is below the P_{crit} value of 82% saturation leading to a decline in oxygen consumption. A similar P_{crit} value (80%) was determined by Jan and Chang (1983) in *H. diversicolor supertexta* Reeve. The P_{crit} for fed *H. tuberculata* was estimated to reach as low as 63% saturation, far lower than the greenlip or Korean Pacific abalone (Gaty and Wilson, 1986). After hypoxic acclimatisation (77 days at 63 to 55% saturation), greenlip abalone decreased their oxygen consumption by one third (Harris *et al.*, 1999) presumably to match oxygen supply and demand. Furthermore, reduced oxygen consumption coincides with a decrease in food consumption, which may partially explain the reduced growth rates during chronic hypoxia (Harris *et al.*, 1999). Decreased growth rates during hypoxia are common among many marine species including clams (Sobral and Widdows, 1997), crabs (Das and Stickle, 1993) and oysters (Baker and Mann, 1992) and this is likely due to the decrease in protein synthesis associated with hypoxia (Hochachka and Lutz, 2001).

Abalone have developed a number of strategies at different physiological levels to cope with hypoxia. Increased gill perfusion allows for increased oxygen uptake while biochemical shifts in haemolymph retain oxygen for oxygen dependent tissues. The muscles have retained considerable capacity for ATP synthesis via PA and anaerobic glycolysis in the face of low oxygen and provide suitable amounts of energy. Meanwhile, the damaging effects of a mismatch in oxygen supply and demand are mitigated by antioxidant enzymes and DNA repair mechanisms. While these physiological adjustments ensure survival during hypoxic events, they come at a great cost to the animal, which limits the scope for growth (Harris *et al.*, 1999). Currently, incoming water is not oxygenated on most farms in Australia and the oxygen saturation in the grow-out tanks is routinely hovering around 80% (unpublished observations). If the P_{crit} value for one of the main commercial abalone in Australia, *H. laevigata*, is at ~ 80% oxygen saturation, then there will clearly be some metabolic limitations in these animals resulting in growth deficiencies. Long term growth studies and comprehensive data sets regarding the metabolic status at these threshold levels of oxygen will be critical for discerning the cost-benefit analysis of aerating the water in the grow-out tanks.

We know little about P_{crit} across species and how this changes with e.g. age, size, diet, temperature, and/or salinity. Understanding critical oxygen levels in a variety of environments will help establish guidelines for water oxygenation on farms to optimise growth rates and prevent oxidative stress. The effects of hypoxia on the foot and adductor muscle metabolism potentially also affect the taste of the abalone (Baldwin *et al.*, 1992). The concentration and composition of taste active components will change based on the metabolic substrates being catabolised in hypoxia vs. normoxia, although this has not been quantified. Given the changes in lactate, tauropine, and free amino acids during hypoxia this area of research presents a novel mechanism for manipulating flavour and palatability. Other research includes understanding how the environment and/or diet can influence these quality factors and whether they can be manipulated to alter, for example, taste; such research will benefit the industry in producing a more consistent product.

IIc. Salinity

In nature, adult abalone are found in fairly stable environments where fluctuations in salinity are rare and may only occur during large rainfall events particularly in tropical environments. Larval and juvenile abalone, however, have the potential to exist in areas of tidal variation

and changes in salinity. On the farm, salinity can change depending on water inlets and tidal flow of coastal waters. The optimal range for growth is at salinity 30 to 35 in many species, but they can survive a range of salinity 25 to 45. As osmoconformers, abalone conform to the osmolality of their environment (Shumway, 1977; Gilles, 1979).

Sudden drops in salinity, often reaching as low as 25 in short periods can occur in many Asian aquaculture systems when the rainy season begins. Optimal growth conditions for their main commercial abalone, *H. diversicolor supertexta* were found to be salinity 30 to 35 (Chen, 1984), but decreases down to salinity 25 can be tolerated (Yang and Ting, 1986). Mass mortality occurs when salinity reaches 20 or lower within 24 h (Chen and Chen, 1999). Juvenile abalone on the other hand, respond well to acclimation to different salinities and can withstand larger changes depending on the temperature and acclimation level (Chen and Chen, 1999). Juveniles reared at salinity 35 can withstand salinity 20 to 45, whereas those acclimated to salinity 25 could tolerate a salinity range of 14 to 33 if the salinity is changed by 2 h^{-1} (Chen and Chen, 1999). Another species, *H. asinina* Linnaeus, could tolerate direct transfer from salinity 32.5 to 20 (Singhagraiwan and Doi, 1992). *H. diversicolor supertexta* were also able to tolerate being directly transferred to salinity 20 and back to salinity 35 over a range of temperatures (20 to 30 °C), but were only followed for 12 h after transfer (Chen and Chen, 1999). Long term tolerance was found to be in the salinity range of 26 to 38 for *H. tuberculata*. Below salinity 26 there was a decline in growth rate (Basuyaux *et al.*, 1998). Edwards (2003) found that survival was variable at salinity 23, but had full survival at salinity 25 in *H. laevigata*, while the upper limit for this species had a similar difference of only 2 (salinity 40 to 42) between full survival and mortalities (Boarder *et al.*, 2001). Similar lower lethal limit was found for *H. kamtschatkana* (salinity 23) (Olsen, 1983). The margin between survival and death appears to be quite narrow in these animals, therefore, a strong understanding of the effects of salinity is key in keeping farmed abalone healthy.

In the wild, and in most aquaculture systems, changes in salinity beyond the lethal limit are rare, however, sublethal changes may have a dramatic effect on growth rate. Declines in growth rate with changing salinity are likely resultant of a reduced heart rate (Nakanishi, 1978) and activity level (Edwards, 2003) and potentially feed intake. Short term exposures to high salinity in *H. cracherodii* and *H. rufescens* induced a stress response as determined by the presence of stress bioindicators by phosphorus nuclear magnetic resonance (P NMR) (Higashi *et al.*, 1989). Heat shock proteins are also elevated in *H. rubra* during both low and high salinity indicating that the animals are stressed (Drew *et al.*, 2001). Greenlip and

blacklip abalone exposed to high salinity appear to be less mobile, difficult to remove from the substrate and had a shrunken foot and blood vessels, but this response was not seen in low salinity stress (Edwards, 2003).

The haemolymph osmolality of *H. diversicolor supertexta* was hypo-osmotic over a range of salinities (35 to 53 mOsm lower than the water) (Cheng *et al.*, 2002). Haemolymph osmolality increased by 69.8% as salinity increased from 23 to 38. Despite large changes in osmolality, the haemolymph stabilised within 3 days over the same range of salinities (Cheng *et al.*, 2002). These changes presumably caused differences in cellular water content of the abalone, and thus changes in weight. This is of particular importance in the abalone market, especially the live shipment of abalone. Changes in cell volume may also have an effect on the immunity and the ability to respond to infections or toxicants in the environment. Elevated salinity caused a reduction in haemocyte response time and reduced the rate at which they moved towards the antigens (Fisher and Newell, 1986). Increased salinity most likely caused swelling of the haemocytes preventing their locomotion within the haemolymph (Martello *et al.*, 2000). Fisher and Newell (1986) postulated that in order for the haemocytes to become active they may be required to be iso- or hyperosmotic relative to the haemolymph. Changes in salinity throughout the year may contribute to the propagation of infections in farmed abalone. In fact, *H. diversicolor supertexta* were more susceptible to *Vibrio parahaemolyticus* when transferred to salinities of 20, 25, and 35 from 30. At salinity 20, all abalone died after 48 h and phagocytic activity and clearance of *V. parahaemolyticus* was decreased (Cheng *et al.*, 2004).

While salinity changes can reduce tolerance to environmental pathogens and toxicants, it can also induce internal damage through oxidative stress. A > 10-fold induction of selenated gluthatione peroxidase (SeGPx) was observed in abalone exposed to a salinity of 25 suggesting that they are oxidatively stressed (De Zoysa *et al.*, 2009). It has been suggested that these large shifts in transcriptional activity may be used as bioindicators of stress in these animals (Abele and Puntarulo, 2004). Oxidative stress is directly linked to respiration and metabolism, and there are significant changes in the metabolic state of abalone exposed to changes in salinity. Phosphoarginine stores in the foot are initially decreased in hypersaline water (salinity 51). In large red and black abalone, however, the PA stores declined but stabilised at approximately 70% of their initial value, while the values in the small red abalone continued to decline (Higashi *et al.*, 1989). When returned to salinity 35, the black and small red abalone returned to pre-treatment levels of PA, although the small red abalone

took twice as long. Interestingly the large red abalone had not fully recovered after 4 h. A similar but quantitatively smaller response was observed when the salinity was reduced by 50%. The ability to recover quickly after stress is advantageous in dynamic environments where multiple stressors or stressors in succession are likely to occur (ie. aquaculture). Understanding the limits to salinity stress in farmed species, and their recovery rates will aid in management decisions and help to prevent mortalities and to understand post harvest treatment technology to optimise the product.

Changes in heart rate, increased stress response and lack of mobility during changes in salinity likely result in large changes in metabolism which would cause decreases in growth rate. Long-term rearing at sub-lethal (within coping range) salinities in abalone requires further investigation particularly as they relate to growth and disease prevention.

IId. CO_2 and pH

Increasing atmospheric CO_2 has become a major concern in the face of global climate change. Oceanic CO_2 levels have been rising and are expected to continue to rise. Increases in dissolved CO_2 in the water are expected to change the pH and interfere with marine chemistry and biological processes. Anthropogenic CO_2 in the atmosphere dissolves into the oceans where it changes carbonate chemistry as well as decreases the pH of the water. Ocean acidification has already dropped the pH an average of 0.1 units (Caldeira and Wickett, 2003; Orr *et al.*, 2005), and it is expected to drop by another 0.3 to 0.5 units by the end of the century (Feely *et al.*, 2009). This is of particular importance to marine calcifiers whose shell development is highly affected by the pH of the water. Moreover, acid base balance of water breathing animals can be highly affected by changes in the pH of the water and uptake of ions at the gills. Recent research has also shown the negative impacts of ocean acidification on fish behaviour. These effects have been shown in a wide array of marine organisms (reviewed in Orr *et al.* (2005); Fabry *et al.*, 2008; Doney *et al.*, 2009; Porzio *et al.*, 2011) and across different developmental stages (see Kurihara and Ishimatsu (2008); Dupont *et al.*, 2009; Talmage and Gobler, 2009).

Abalone larvae are particularly sensitive to changes in pH and the majority of research has focused on this life stage with little work on juveniles and adults. Harris *et al.* (1999) conducted a long term assessment on two species of abalone, greenlip and blacklip, and their response to low pH. Both species showed a significantly reduced specific growth rate (length

or whole mass). pH values of 7.78 and 7.93 for greenlip and blacklip, respectively, induced a 5% reduction in growth rate, while 50% reduction in growth rate was found at 7.39 and 7.37. At pH 6.79 survival was significantly reduced in both species and respiratory rate was also decreased. At pH 7.16, kidney and gill abnormalities develop likely contributing to low survivorship. While this study investigated the effects of pH on abalone physiology, it is limited in its impact on ocean acidification in that the pH was controlled via HCl and NaOH, and not by CO_2. Given that CO_2 drives changes not only in pH, but also in carbonate chemistry it will be imperative to do long term studies using CO_2 control to determine the effects in the wild and on the farms.

Larval life stages are considered to be the most vulnerable period and are key to understanding population dynamics (Heath, 1992; Gosselin and Qian, 1997). In a study comparing present day and near future ocean CO_2 values, it was found that the fertilization and hatching rate decreased in *H. discus hannai* after 15 h of high CO_2 (> 1650 µatm) exposure and that larval SL was significantly lower and the malformation rate was increased after 75 h (Kimura *et al.*, 2011). Abalone secrete a more soluble $CaCO_3$ and aragonite during the larval stages for shell growth (Weiss *et al.*, 2002; Jardillier *et al.*, 2008) which may make them more susceptible to low pH. 40% of *H. kamtschatkana* larvae developed shell abnormalities at 800 ppm CO_2 after 8 days of exposure. At 1800 ppm, all abalone had shell abnormalities or had no shell at all (Crim *et al.*, 2011). Despite this, all the abalone survived in this experiment. Yet, in the wild shell-less abalone would be more susceptible to predation and survival would not be expected (Hickman, 2001). For farmed species, however, changes in CO_2 and water pH are less of an issue during the larval phase as water quality is strictly monitored during this time.

The effects of CO_2 on chemosensory signalling that occur in fish (Munday *et al.*, 2009) may also be important for larval settlement in abalone although this has not yet been tested. It is unclear if these adverse effects will persist into the juvenile and adult life stages, even if they are more tolerant. Long term studies are required to determine how abalone reared in high CO_2 as larvae will respond as adults in terms of growth and survivorship. A pH of 7.87 significantly decreased the thermal tolerance for some developmental stages (pre-torsion and late veligers) of *H. rufescens* (Zippay and Hofmann, 2010). There were no changes, however, in the expression of two genes involved in shell formation, indicating that the effect of CO_2/pH is more likely to be at the protein and enzymatic level in these animals (Zippay and Hofmann, 2010). In contrast, a large genomic study on single nucleotide polymorphisms

(SNPs) in *H. rufescens* found a large number of genes in the mantle involved in biomineralization, energy metabolism, heat-, disease- or hypoxia-tolerance, that changed upon exposure to CO_2 (De Wit and Palumbi, 2013). Therefore, the effects of CO_2 and pH may have a more wide-reaching effect on abalone besides the growth of the shell, particularly in adults.

There has been debate about the extent to which marine organisms will be able to cope with ocean acidification given the substantial variability among species and between life stages. Using meta-analysis methods, several authors have combined the results of published data on all marine life to establish the threat of ocean acidification in the future. Some authors suggest that marine life will be able to cope with the slow rate of change in the oceans (see Hendriks *et al.* (2010); Dupont *et al.*, 2010), while others believe they are vulnerable (Kroeker *et al.*, 2010). Both groups, however, agree that marine calcifiers are the most at risk. These meta-analyses have been completed on single exposure to one risk (CO_2), and do not include the synergistic effects of other environmental stressors such as warming and hypoxia. In many cases, the exposures are often short term and do not take into account the ability of animals to acclimate if the exposure is gradual rather than sudden. Recent research in other molluscs (Parker *et al.*, 2012) has found that exposure of adults to climate change conditions, including high CO_2 may have a protective effect on developing larvae through maternal or epigenetic effects. In particular, larvae of oysters exposed to high CO_2 during reproductive conditioning grew faster and larger when reared in high CO_2 versus those whose parents were not exposed (Parker *et al.*, 2012). These types of pre-conditioning and multi-generational experiments would benefit the abalone industry which can sometimes face large fluctuations in various environmental factors. If broodstock could be acclimated to high CO_2 conditions then the larvae may develop quicker and be less sensitive to environmental changes as adults. There has been little research conducted on farms to determine the CO_2 levels and pH within grow-out systems. Fundamental knowledge in this area will help to predict future growing conditions on the farm, and if adults will be susceptible to the same effects as wild adult abalone.

IIe. Synergistic Effects of Climate Stressors

Environmental disturbances rarely involve only a single stressor. Future climate change scenarios predict increases in temperature, increases in CO_2, and more frequent extreme weather events, which combined, will impact all of the above environmental parameters.

Currently, the majority of published research describes the effects of singular stressors and have provided a strong foundation for future research on the synergistic effects of environmental stressors. It has been predicted using other aquatic species that multiple stressors will reduce physiological performance (e.g. metabolic rate, swim performance, growth) and the maximal coping range (critical level) for the individual stressors (Pörtner and Farrell, 2008). For example, increases in temperature drive increases in metabolic rate and thus oxygen demand. Concurrently, increases in temperature decreases the dissolved oxygen in water thereby limiting oxygen to meet the demands of the animal, and this oxygen limitation reduces the thermal tolerance (known as the oxygen- and capacity-limitation of thermal tolerance (OCLTT) (Pörtner and Knust, 2007)). While abalone can survive a large range of temperatures and dissolved oxygen independently, their ability to survive (or grow) will be further limited during simultaneous increases in temperature and decreases in oxygen. The same will likely be true for other combinations of environmental stress. Furthermore, the addition of farm stressors will exacerbate the limitation on physiological performance and growth.

III. Physiological response to farming

Abalone farms create a set of stresses which are generally unseen in the wild and these stresses alone or in combination with environmental stress can lead to high mortality and/or low quality product. Culturing animals has two major requirements; adequate nutrition and waste removal. These requirements are dependent on the stocking density which is normally extremely high. High density culture (and subsequent high feeding rates) without adequate waste removal and water turnover results in ammonia build up in the water and increased prevalence of disease. Culturing animals also adds the additional stress of human interaction and handling whether it is for size grading, checking for disease or moving to new areas of the farm. While these stresses are often mitigated as best as possible, they do still exist, and the results of this can be lower growth rates, low quality products, or if left unchecked, high mortality rates.

IIIa. Ammonia

Nitrogenous waste from cultured aquatic animals is largely in the form of ammonia (NH_3, NH_4^+) (Spotte, 1979), a toxic waste product of amino acid metabolism. In the natural oceanic

environment of an abalone, ammonia concentrations rarely reach toxic levels. Yet, in semi-enclosed densely packed aquaculture facilities ammonia can easily reach levels that affect physiological functions and ultimately growth. Ammonia is not only produced by the animals, it also comes from heterotrophic bacteria in the water which consume waste, uneaten food and dead animals (Rheinheimer, 1991). In the aquatic environment ammonia exists in equilibrium in its free form (NH_3), ionic form (NH_4^+) and hydrogen ions (H^+) which makes its concentration dependent on pH and temperature (Colt and Tchobanoglous, 1976). The concentration of NH_3 increases as temperature or pH increases and it is this form which is the most toxic because it readily diffuses across cell membranes (Thurston *et al.*, 1981).

Ammonia toxicity has been well studied in aquatic vertebrates (Randall and Tsui, 2002), and has also received particular attention in aquacultured animals (Basuyaux and Mathieu, 1999; Hargreaves and Kucuk, 2001). Ammonia concentrations can vary throughout the day as well as between tanks which can lead to stress in the animals. Ammonia affects many biological functions in marine organisms and can cause changes in tissue structure of the gill (Smart, 1976), cellular function, blood chemistry, osmoregulation, immune function and disease resistance, reproduction (Colt and Armstrong, 1981; Russo, 1985; Jeney *et al.*, 1992), respiratory function and MO_2 (Smart, 1976; Chen and Lai, 1992; Chen and Lin, 1992). Given the broad effects of ammonia on physiological functions chronic exposure can also lead to decreased growth rates and potentially mortality in catfish (Hargreaves and Kucuk, 2001).

Studies on the effects of ammonia on abalone specifically have been relatively limited to date. Basuyaux and Mathieu (1999) investigated the growth rate of *H. tuberculata* at increasing NH_3 concentrations and found that there was reduced growth in terms of length and weight above 0.5 mg L^{-1} accompanied by a decrease in food consumption. A similar result was found in *H. laevigata*, but they were approximately 10x more sensitive (5% reduction in growth at 0.05 mg L^{-1}) (Harris *et al.*, 1998). The decrease in growth rate was associated with a decrease in food intake and an increase in metabolic rate which would limit energy for growth. Acute exposures at a similar level, however, do not appear to affect growth rates of *H. laevigata* and *H. rubra* (Hindrum *et al.*, 2001). Yet, in a more long term exposure, the concentration to reduce growth by 5% was found to be 10 x lower still (0.005 mg L^{-1}) than that found in the study by Harris *et al.* (1998) (Huchette *et al.*, 2003b). This was attributed to the number of animals tested and the duration of the experiment (15 days, $n < 200$, Harris *et al.* (1998) vs. 4 months $n = 1800$, Huchette *et al.* (2003b)). The age/size class of the abalone likely also played a role in their ammonia tolerance. The South African abalone, *H. midae*

becomes more tolerant to acute exposure to ammonia with increasing size (Reddy-Lopata *et al.*, 2006). In the same study however, it was shown that juvenile abalone specific growth rate was almost 60% lower during a chronic exposure. Growth rate was also lower in *H. fulgens* when exposed to light (Searcy-Bernal *et al.*, 2003), and this is postulated to be a result of increased ammonia production during light exposure (Ahmed *et al.*, 2008a).

To mitigate these issues, land based farms must balance water flow for physiological and economical constraints (Evans and Langdon, 2000). Flow rates must take into account the density, bacterial load of incoming water, faecal excretion rates, feeding frequency and volume, and potentially dietary protein concentrations which could lead to increased ammonia excretion. New research using multi-diet feeding strategies with varying levels of protein for different ages and different grow-out temperatures is promising for increased growth potential (Stone *et al.*, 2013), but as yet there have been no investigations into the increased ammonia load with these diets which could potentially negate the growth effects. Furthermore, at high temperatures ammonia becomes increasingly toxic, but there has been no research into the interactive effects of ammonia and temperature on abalone. Ammonia excretion is significantly increased at higher water temperatures (Park *et al.*, 2009) and perhaps ammonia toxicity at high temperatures is playing a role in summer mortality.

IIIb. Density

The density of abalone in nature can be low while in aquaculture, target species experience high stocking densities for economical reasons (Webber and Riordan, 1976). The current commercial stocking density for Australian abalone is 40 kg m^{-3} (Freeman, 2001). At high stocking densities growth of individuals is often below the optimum rate, but maximal biomass gain counterbalances the loss and ultimately yields the most profit (Mgaya and Mercer, 1995; Wassnig *et al.*, 2009; Wassnig *et al.*, 2010). There is ongoing research on profitability with regard to stocking density and growth of abalone in various culture systems (Badillo *et al.*, 2007; Park *et al.*, 2008; Wu *et al.*, 2009; Minh *et al.*, 2010; Encena *et al.*, 2013; Qi *et al.*, 2013; Wu and Zhang, 2013).

Douros (1987) supplied the first evidence that abalone will stack on each other if the number of individuals in a given area is too high (Douros, 1987). The observed behaviour is a response driven by a lack of attachment space. In a restricted area individuals then compete for shelter and food, which leads to reductions in growth (Huchette *et al.*, 2003b), either

recognised as shorter SL (Huchette *et al.*, 2003a; Wu *et al.*, 2009; Jarayabhand *et al.*, 2010), lower weight gains (Park *et al.*, 2008; Wassnig *et al.*, 2009; Ahmed *et al.*, 2013) or both (Mgaya and Mercer, 1995; Marsden and Williams, 1996; Capinpin *et al.*, 1999; Fermin and Buen, 2002; Badillo *et al.*, 2007; Daume, 2007; Wu *et al.*, 2009; Minh *et al.*, 2010; Encena *et al.*, 2013). Indeed, the feeding rate of abalone has been shown to be inversely related to density (Marsden and Williams, 1996; Fermin and Buen, 2002; Lloyd and Bates, 2008; Ahmed *et al.*, 2013). A common explanation for this relationship is that individuals at the bottom of a stack are not able to move unless those from the top descend (Mgaya and Mercer, 1995). Stacking, and thus stocking density, does not affect the feed conversion rate (FCR) of abalone (Capinpin *et al.*, 1999; Fermin and Buen, 2002; Park *et al.*, 2008; Ahmed *et al.*, 2013). Also Encena *et al.* (2013) found no influence of density on FCR of *H. asinina* when individuals were grown in multitier trays or boxes. Yet, they reported an inverse relationship when abalone were reared in mesh cages.

Decreased growth rate at high densities may indicate that they experience higher physiological stress (Gaty and Wilson, 1986; Marsden and Williams, 1996; Huchette *et al.*, 2003a; Day *et al.*, 2004). Even at the lowest tested density in the laboratory, growth rates of abalone were only half as high as those reported from the field (Marsden and Williams (1996) and references therein). It has been shown that high densities affect water quality, in terms of ammonia and oxygen concentrations, lead to an increase in bacteria levels and impede feeding; all of which are known to interfere with the metabolism of abalone (Harris *et al.*, 1999; Huchette *et al.*, 2003b; Theil *et al.*, 2004; Macey and Coyne, 2005; Lloyd and Bates, 2008). A positive correlation between mortality and density has been reported for several abalone species when densities exceeded a certain threshold (Mgaya and Mercer, 1995; Park *et al.*, 2008; Wassnig *et al.*, 2009; Minh *et al.*, 2010; Vivanco-Aranda *et al.*, 2011; Ahmed *et al.*, 2013; Encena *et al.*, 2013). Critical and optimal density values are dependent on culture systems, developmental state of the individual, the species and abiotic conditions (Mgaya and Mercer, 1995; Ahmed *et al.*, 2013; Fig. 2.2). This critical threshold was probably not reached in studies which found no relation between mortality and density (Capinpin *et al.*, 1999; Jarayabhand *et al.*, 2010). Indeed, abalone densities used on aquaculture farm exceed those used in laboratory experiments by far, in being 10000 individuals m^{-2} on the farm and e.g. 500 to 1000 individuals m^{-2} in the laboratory for abalone of 7 to 15 mm SL (Heasman and Savva, 2007; Encena *et al.*, 2013).

The number of individuals per unit area affects larval settlement, post-larval survival and growth, and thus influences later juvenile performance. Survival rates of abalone during the first five months of their development are estimated at only 0 to 10% in wild populations. The preferred substrate for settlement might be limited, which results in intraspecific competition at high larval numbers while predation further causes mortality numbers to increase (McShane, 1991). On the other hand, in culture, food is available in excess and predation is not existent but survival is still low (22%) (Daume *et al.*, 2004). Stocking density on farms negatively affects settlement rate (Courtois De Viçose *et al.*, 2010; Daume *et al.*, 2004) and high densities of settled individuals in turn negatively affect post-larval growth (Daume *et al.*, 2004; Day *et al.*, 2004) and survival (McShane, 1991; Daume *et al.*, 2004; Day *et al.*, 2004; Courtois De Viçose *et al.*, 2010). Possible reasons for these density effects include impaired health due to higher susceptibility to bacteria/viruses, or complex interactions of environmental factors, such as variable oxygen and carbon dioxide levels resulting from photosynthesis of algae (food) and respiration (Day *et al.*, 2004). Daume *et al.* (2004) concluded that it is important to control stocking density to optimise profit in aquaculture as early-life growth influences juvenile performance. According to Mgaya and Mercer (1995) sorting of abalone with respect to their size would improve production as growth of small individuals is increased when they are cultured with same-size conspecifics. Both studies do not advise culling, as its costs and gains are not fully understood. Indeed, sorting might have a more detrimental effect, at least at later life-stages, as handling decreases the immune function (see handling section) (Hooper *et al.*, 2011). Furthermore, Wu *et al.* (2009) did not suggest grading as their results did not show significance. This study, however, was conducted with densities (12% of available tank floor area) far below those used in industry (60%) (Wu *et al.*, 2009). Yet, it showed that small abalone are more density-sensitive than larger ones. In contrast, Jarayabhand *et al.* (2010) predicted that growth of larger individuals is more susceptible to increasing densities than those of smaller individuals. Hence, they suggest grading as a regular practice in abalone aquaculture. The underlying mechanisms remain unclear as the physiological responses of abalone to high densities are relatively unexplored. Many of the issues associated with high density culture are known to affect the metabolism and growth rate of abalone, but these responses are likely species and size specific making it difficult to determine the optimal density for growth and profitability. In Fig. 2 growth rate data are shown at the species level of small (6 to 24 mm) and large (29 to 60 mm) abalone at different densities confirming the specificity of this variable. It appears that small and large tropical *H. asinina* are more susceptible to small increases in density

when compared to other more temperate species. Interestingly, the 29 to 60 mm greenlip/blacklip hybrid abalone follow the same pattern, but there are limited data on the effects of the pure species. For the two abalone species (*H. corrugata* and *H. tuberculata*) tested with densities above 500 individuals m^{-2}, there appears to be no loss in growth rate (g or mm $month^{-1}$) with increasing density (Mgaya and Mercer, 1995; Badillo *et al.*, 2007) suggesting that at least these species can be cultured at extremely high density with no loss in growth. It would be interesting to determine if the Australian species behave in the same manner at higher stocking densities.

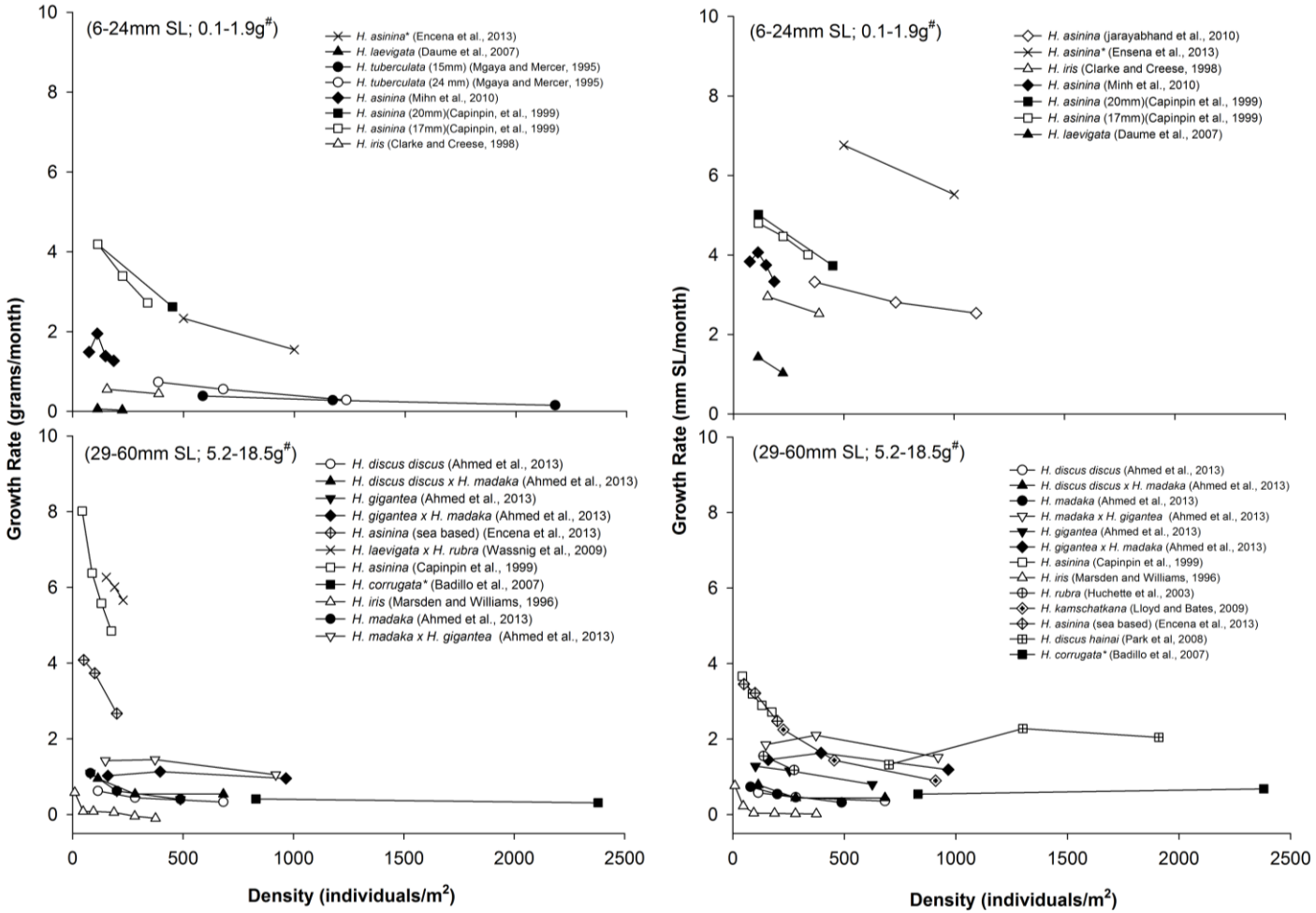

Fig. 2.2: The effect of density on growth rate (mm shell length (SL)/month or g/month) in two size classes (6 to 25 mm SL or 25 to 60 mm SL) of various abalone species. * = Average growth rate from 3 different grow-out protocols. # = Average weight range; weight not indicated in all references.

IIIc. Handling

Current abalone rearing techniques require handling, although it is minimised as much as possible as it can cause high mortalities (La Touche *et al.*, 1993; Mgaya and Mercer, 1995; Hooper *et al.*, 2011). In the hatchery, handling of individuals to induce spawning includes their detachment from the substrate and exposure to air, ultraviolet light and increased temperatures (Al-Rashdi and Iwao, 2008). As abalone grow they are transferred to adjust stocking densities, for individual tagging, pearl seeding, relocation of facilities, or grading (Aquilina and Roberts, 2000; Chacon *et al.*, 2003; Hooper *et al.*, 2011). During transfer individuals need to be detached from the surface and are exposed to air and varying temperatures. Finally, some individuals are sold and transported live. During live transport individuals are starved for several days, graded, exposed to air for up to 48 h and to varying temperatures ranging between 4 and 32 °C (Fallu, 1991).

Protocols about handling practices in the hatchery are abundant (Ebert and Houk, 1984; Ritar and Elliott, 2002; Al-Rashdi and Iwao, 2008). Li (2004) published a detailed protocol to establish an abalone farm for *H. rubra* and *H. laevigata*, containing information about broodstock collection and maintenance, spawning and fertilisation methods, hatch-out and larval rearing as well as grow out maintenance for juveniles off plates (Li, 2004). The impact of the handling processes on either broodstock or early life stages is relatively unknown despite the fact that precipitate spawning occurs when abalone are exposed to stressful conditions (Freeman *et al.*, 2006). Farmers take advantage of this behaviour for spawning by exposing their broodstock to changing temperatures and UV irradiated water (Grubert and Ritar, 2005; Daume, 2007). The number of successful spawning events did not change when handling was further increased by fortnightly chipping (removal from the substrate) for weight and length measurements. Yet, the number of eggs produced by the female was twice as high when not handled compared to handled individuals, though this result was presented with insufficient numbers of replicates to reach statistical significance (Freeman *et al.*, 2006).

The detachment of juveniles from the substrate is difficult as abalone clamp their shell to the bottom when disturbed. Changes in water temperatures or exposure to air can be used to dislodge abalone from the surface (Aquilina and Roberts, 2000), however, the most common methods in aquaculture to remove abalone are chipping or anaesthetising (Hooper *et al.*, 2011). The former is a process during which a blunt spatula is quickly put between foot and substrate so that the individual can be levered off the substrate (Robinson *et al.*, 2013). It is possible that individuals are cut during chipping and as a result they might bleed to death if

the muscle is damaged deeply (Chacon *et al.*, 2003). Further, it has been reported that this method can lead to higher rates of mucus production and thus a loss in energy (McCormick *et al.*, 1994; Chacon *et al.*, 2003). Various chemicals, such as benzocaine (BZ), magnesium sulphate (MS), or phenoxyethanol (PE) are used as anaesthetics (Mgaya and Mercer, 1995; Chacon *et al.*, 2003; Robinson *et al.*, 2013). Chemicals have been reported to be successful in removing abalone from surfaces with both high and quick recovery rates, which were mainly measured as strength of reattachment and survival rates (Hahn, 1989; Aquilina and Roberts, 2000; Al-Rashdi and Iwao, 2008; Bilbao *et al.*, 2010). Yet, anaesthetics have been shown to cause more stress than manual removal from the substrate (Chacon *et al.*, 2003). Knowledge about physiological recovery, however, remains scarce.

Anaesthetising is assumed to have the highest impact on physiology when compared with other handling practices (Hooper *et al.*, 2011). Oxygen consumption rates, growth rates, immunity and the cardiovascular system of abalone respond to anaesthetics and need several days to return to baseline levels (Edwards *et al.*, 2000; Chacon *et al.*, 2003; Sharma *et al.*, 2003; Hooper *et al.*, 2011). Haemocyte counts, antibacterial activity, phagocytic rates and lysosomal membrane integrity of *H. laevigata* and *H. rubra* were more affected in animals that were transferred and removed with anaesthetics than in those which were chipped (Edwards *et al.*, 2000). Irrespective of the removal method, abalone had a weaker immune system for three to five days post handling than prior to transfer (Hooper *et al.*, 2011). Also MO_2 of abalone needs up to five days to recover from chemical application, while only one day is needed after mechanical removal (Edwards *et al.*, 2000). Substances, such as MS, BZ and ethanol caused MO_2 suppression while others, such as potassium chloride (KCl), Aqui-S and clove oil induced MO_2 stimulation (Edwards *et al.*, 2000; Chacon *et al.*, 2003). Clove oil is a natural anaesthetic attained from dried flower buds of *Eugenia caryophyllata* Thunberg, yet statements about its application are equivocal (Edwards *et al.*, 2000; Bilbao *et al.*, 2010). Bilbao *et al.* (2010) reported clove oil as suitable for the removal of *H. tuberculata coccinea* Reeve, even if it is unsuitable for pearl seeding due to insufficient relaxation of the adductor muscle. Yet, Edwards *et al.* (2000) used similar concentrations of the oil but they reported enhanced mortalities of abalone and no recovery of increased MO_2 values after six weeks post treatment. Also growth rates remained suppressed six weeks after the application, though this was independent of the type of anaesthetic, natural or chemical. Further, the heart rate of abalone has been shown to halve when individuals were treated with sodium pentobarbitone (Sharma *et al.*, 2003). Ongoing treatment after individuals were released from the substrate

lead to further reductions in heart rate or resulted in irregular rhythms. After the chemical was removed from the water individuals were quickly able to right themselves while heart beats increased compared to rates prior to the application and took eight days to reach baseline levels again (Sharma *et al.*, 2003).

New research will hopefully lead to establishing the best protocols to reduce stress during handling while also maintaining healthy abalone.

IIId. Exposure to air

Exposure to air occurs during many farming procedures including larval movement to grow out tanks (during transfer of individuals on mesh screens), size grading, and live transport. Abalone remain metabolically active in the air, albeit at a much lower rate (Baldwin *et al.*, 1992), and this physiological feature has been exploited for live shipment of these animals over long periods. Exposure to air, however, is not without consequence, as it can affect not only mortality rates, but metabolic/growth rates, immunity, metabolite concentration and water content and thus, taste and texture.

During exposure to air the gills collapse effectively reducing the ability to extract oxygen and therefore abalone begin to rely on anaerobic metabolism and phosphate reserves for energy production. *H. kamtschatkana* showed decreased levels of arginine phosphate in the adductor muscle but not in the foot after 16 h of exposure to air (Donovan *et al.*, 1999). Yet, both tissues exhibited significant increases in tauropine and lactate, and this finding was echoed in *H. iris* and *H. australis* Gmelin (Baldwin *et al.*, 1992; Wells and Baldwin, 1995). Furthermore, ATP levels and energy charge (ATP to ADP/AMP ratio) decreased in both *H. iris* and *H. australis*, but the extent of these changes was dependent of size. Wells and Baldwin (1995) suggest that the smaller sized abalone accumulate higher concentrations of the metabolites and are highly energetically stressed which may affect mortality rates significantly more so than in larger abalone when transported live. Other sensory metabolites (taste active components) have been investigated including glycogen, free amino acids (FAA) and nucleotides in *H. diversicolor* Reeve during several days of exposure to air at 5, 15, or 25 °C (Chiou *et al.*, 2002). Glycogen, an energy storage molecule, declined during exposure to air at all temperatures, but the rate was slower at 5 and 15 °C compared to 25 °C. Taste active amino acids (taurine, arginine, glycine, glutamic acid, alanine) in these abalone increased by 10 to 100% of their initial values suggesting an increase use of phosphate stores

and protein degradation. Whilst these taste-active amino acids may increase palatability in the short-term, they are overridden by the subsequent increase in volatile basic nitrogen from decomposition shortly after. Metabolites in the tissue are further concentrated by water loss during exposure to air. Water loss can range from 3 to 15% of total body weight and is mainly lost through evaporation and mucus production (James and Olley, 1970; Vosloo and Vosloo, 2006). Haemolymph volume also decreases by 30% which results from a redistribution of water to the tissues (Vosloo and Vosloo, 2006). Most extended exposures to air occur in adult abalone, but can also happen during larval transport from hatcheries to grow out facilities. After 10 and 36 h in air, *H. rufescens* larval survival was 88 and 50%, respectively, compared to water transport which had 97 and 63% survival. The transport method had no significant effect on larval settlement upon arrival (Pereira *et al.*, 2007). These data suggest that larvae may be less sensitive to exposure to air than juveniles or adults.

To mitigate the effects of exposure to air during live transport several procedures are used. Firstly, animals are starved for 2 to 3 days to prevent defecation (Sales and Britz, 2001), but there is also evidence that starved abalone survive better than fed animals (Watanabe *et al.*, 1994). Supplemental oxygen and ice packs for temperature regulation have also improved survival rates (Bubner *et al.*, 2009; Buen-Ursua and Ludevese, 2011). Changes in temperature during live transport would be expected to have similar effects as it does in water; i.e. changes in metabolic rate, growth rate, anaerobic metabolism, cellular damage, and immune function. Indeed, lysosomal membrane stability declines with increasing air temperature during transport and takes longer to recover upon re-immersion (Song *et al.*, 2007).

Recovery times post exposure to air and the effects of various re-immersion conditions on abalone survival and taste properties remain elusive. This is a potentially lucrative area of research as this type of data would help to prevent mortalities during and after live shipment and also to establish the best arrival conditions for recovery to produce the best tasting abalone.

IIIe. Nutrition and Diseases

Nutrition and diseases are two of the most important biological and economical issues facing abalone farmers. Adequate nutrition is undoubtedly the main factor in ensuring fast growth while disease prevention reduces mortalities and produces higher quality animals. These areas of research are extensive and have been reviewed previously (Fleming *et al.*, 1996;

Handlinger *et al.*, 2006). Therefore only a brief overview of these topics with respect to the environment and how nutrition may be used combat the effects of temperature or disease are presented.

An early review of abalone dietary requirement was composed by Fleming *et al*. (1996) and gives a comprehensive outlook into formulated abalone feed including major constituents and their sources, vitamins and minerals, binders, energy requirements, digestibility, attractants, stability and decomposition (Fleming *et al.*, 1996). This was again briefly updated in 2004 (Sales and Janssens, 2004). Similar conclusions were drawn by both studies; that there is not enough information on the nutrient requirements of abalone. Secondarily, there are still many publications which rely on unreliable methods for food consumption measurements as well as growth measurements (ie. SL only and using a correlation between length and live weight). These measures make it difficult to accurately assess the feed conversion ratio of the diets.

Aside from the lack of details on nutrient requirements of abalone, there is also very little research on dietary needs with respect to the environment. The farm environment can be challenging for the animals, particularly in temperate regions where fluctuations in temperature occur daily and seasonally. Whilst the first priority remains to establish nutrient requirements, the next logical step will be to understand how feeding, digestion and energy conversion are influenced by environmental stress such as temperature, oxygen saturation, CO_2 and air exposure. In fact, there has been some preliminary investigations in other species such as sea urchins (Matson *et al.*, 2012), lobsters (Dall, 1974) and crabs (Dumler and Terwilliger, 1996). Recent work in abalone has investigated the effects of dietary requirements at elevated temperatures (David Stone, Flinders University, pers. comm.) (Green *et al.*, 2011) that indicate that optimal growth can be achieved with higher energy content and the optimal amount of protein in the diet. Studies like these will be important for formulated feeds to match environmental conditions to ensure optimal growth year round. Additionally, supplementing food with immunostimulants and/or cessation of feeding during warmer weather will help prevent diseased induced mortality that is associated with warmer water temperatures. Indeed, fed abalone at higher temperatures had higher prevalence of Rickettsiales-like prokaryote which causes withering syndrome in red abalone than those which were starved (Braid *et al.*, 2005). There are a suite of other diseases (Handlinger *et al.*, 2006) found in farmed abalone, many of which may also be exacerbated by high temperature. The effects of other abiotic stresses on abalone disease persistence and aetiology are unknown.

There needs to be some long term chronic exposures to sub lethal stresses because most animals will not feed when overly stressed, as feeding during stressful events may also kill them. Conversely, how does starvation affect the abalone? Abalone experience periods of starvation throughout their life on farms mainly during purging periods before shipment, and during/after shipment itself. These periods of starvation undoubtedly modify their metabolism and most likely induce changes in tissue chemistry and potentially taste and texture. Pre-shipment diets may be an interesting area of research to ensure survival and preserve high quality of the meat.

IIIf. Sexual Maturation and Spawning

Sexual maturation and spawning in abalone are complex and regarded as major issue in commercial abalone stocks as reviewed in Botwright *et al.* (2014). In general, during sexual maturation energy is diverted into gonad development and gamete production thereby limiting growth. Gonad tissue is regarded as unfavourable on the market, thus there is a drive to control sexual maturation and unwanted spawning in commercial abalone. Furthermore, spawning interferes with immune function, likely through loss of energy reserves, and can contribute to increased pathogen susceptibility and mortality (Travers *et al.*, 2008). Spawning can be induced by increases in water temperature, therefore the issue of spawning becomes greatest during summer months. It has been proposed that "summer mortality" may be due to a combination of high temperatures and uncontrolled spawning of commercial stocks (Botwright *et al.*, 2014) which likely leaves them energetically deprived and susceptible to pathogens, heat stress, hypoxia, and handling, amongst others. Protocols have been established for the timed induction of spawning of broodstock, but at present there is limited control for the prevention of sexual maturation and/or spawning of commercial stock. Recently, Botwright *et al.* (2014) have begun to establish the molecular mechanisms signalling maturation which is an important step in understanding how we can control and prevent spawning in commercial abalone to reduce mortality and maintain production of quality abalone throughout the year.

IV. Conclusions and perspectives

IVa. Impacts of climate change on farming

The International Panel on Climate Change predicts that air and sea surface temperatures will continue to rise with estimates of as much as 3 °C by 2100 (IPCC, 2013). Moreover, the

frequency of extreme weather events and the severity of *el niño* and *la niña* weather patterns are predicted to increase. Together, this suggests that there will be hotter days for longer periods, increases in maximum summer air and water temperatures and associated decreases in water oxygen saturation, and larger fluctuations in salinity from extended periods of drought or heavy rainfall. The individual effects of these stresses on abalone growth and physiology have been discussed, but what of their synergistic effects? All environmental challenges incur an energetic cost, whether preventative or restorative of the damaging effects of the stress. Most farmed aquatic species have a finite amount of resources/energy that they can invest in various physiological functions, meaning that the more energy spent on maintenance or recovering during/after stress, the less energy is available for growth. Simply put, the greater the frequency and/or duration of stressful events on the farm, the longer it will take for the abalone to reach market size and a greater investment of time and money will be required.

It has also been noted that almost all of the environmental and farm stresses discussed here affected abalone immunity in some way. Disease outbreaks have already become an issue on many Australian abalone farms, and it is predicted that the frequency of these outbreaks may increase. As temperature rises and salinity fluctuates for longer durations, we expect that immune function will be compromised contributing to increased infection rates and spread throughout the farms. Furthermore, recovery periods from infections may be longer if environmental conditions are not ideal. Often associated with stressful events is the increased release of ammonia. Given its toxicity at high temperatures this will compound the effects of these stresses. The ability to combat all of these issues will require energy and thus, oxygen. High density culture in shallow raceways faces the largest risk in this respect. As water enters the system at increasing temperatures there may already be a loss of oxygen. As the water passes through the raceways there will be a further loss of oxygen from use by the animals, but the water will also be expected to heat up in accordance with the air temperature further lowering the maximum dissolved oxygen level. Without adequate water oxygenation abalone face severe risk of the damaging effects of ROS and also the inability to generate enough energy for growth and fighting off infections.

There are several measures that can be taken to combat these potential pitfalls in the abalone industry. Of importance is the ability to utilise the natural variation within the species through well designed and managed selective breeding programmes, which take into account information from physiological and other challenge tests. There will also need to be

investments in water quality control and management, particularly for adult abalone in grow-out tanks. It is unclear when and to what extent these improvements will be required, but realistic climate predictions by the IPCC indicate that it could be in the very near future. While average temperatures are unlikely to be an issue going forward, it will be the extreme weather events that pose the greatest risk. If investments are made now for establishing the most suitable breeding populations, and the ability to control water quality, careful environmental and biological monitoring may allow farmers to be able to predict when interventions are required, and restrict costly control measures to those times of the year when water quality is poor. Such infrastructure modifications may include increasing water flow, oxygenating the water, more efficient ammonia scavenging and salinity and temperature control. Before these modifications are made however, there is a wide range of research that remains to be conducted.

IVb. Future Research Directions

There is currently a large body of research on the effects of environmental stressors on whole animal physiology i.e. metabolic rate, growth rate, mortality rate, and behaviour, but an understanding of the mechanisms associated with these changes are limited. Indeed, if organismal function is negatively affected, an understanding of processes at cellular, molecular and genetic levels will aid in identifying where the potential adaptive limitations are. It will also provide specific targets for intervention and management decisions that will promote health and profitability within the industry.

There exists a large area of research into the mechanisms of intergenerational effects of environmental exposures in other animals, and it is likely that epigenetics will be the next major advancement in the selective breeding of abalone and other cultured animals. We currently know very little about how the effects of the environmental conditions on broodstock will affect the next generation. An understanding of the genetic components of temperature acclimation, for example, will enable geneticists to pinpoint target genes or areas within the genome that can be selected to confer a greater tolerance to thermal stress. In the same respect, there are advancements in the study of critical developmental windows during which embryos or juveniles are “pre-conditioned” to particular stresses which shapes the developmental trajectory and ultimately the adult phenotype (Burggren and Reyna, 2011).

There are many examples within this review highlighting differences in the effects of stress during different life stages of abalone. It will be key in future experiments to examine the effects of the environmental and farm stressors at all life stages to generate a comprehensive outlook on these effects. There exists an issue within the current literature and research on abalone in that there have been no defined indicators of life stages beyond the larval stage. Often abalone are referred to by their size or year class, however, within the literature a "juvenile" can range from 2 to 60 mm, for example. There are many studies confirming differences between juveniles and adults, yet we currently have no delineation between these two life stages and have no measure of predicting how long these differences may last. The "size at age" metric can often be misleading as growth rate is highly variable and dependent on many factors including if the animals are from wild or hatchery stocks and may also be species specific. Most of the developmental literature states that abalone reach sexual maturity within 2 to 3 years or at a particular size. Yet, a 1 year range is large with respect to the changes we see in different sized abalone. As a distinct example, Vosloo *et al.* (2013a) found differences in hypoxia tolerance between juvenile and adult abalone with only a 24 mm difference in SL (41 mm vs 65 mm), but there is still debate as to when these animals reach sexual maturity, and this can vary between the sexes (Wood and Buxton, 1996; Freeman, 2001) (no sex or maturation stage were given in the Vosloo *et al*. (2013a) study). Given the wide range of size and age for juveniles or adults and the lack of consistent use of metrics within the literature (SL, shell width, shucked weight, whole weight, age) it is difficult to compare studies and make conclusions on the effects of environmental or farm stressors on these two life stages. Having a clear definition of the "juvenile" life stage will enable farmers to use available research to refine rearing practices specifically for those abalone which may differ from "adults".

The end of the juvenile stage may simply be sexual maturity but this is difficult to ascertain without rigorous sampling and histological analysis. With advances in genetic testing it may be possible in the future to determine maturity through mucus samples. Sexual maturation and spawning can decrease growth rates or increase undesirable growth (ie. gonads) and reduce quality and health of the commercial stock (Travers *et al.*, 2008; Botwright *et al.*, 2014). Spawning is an energetically costly activity that requires long periods for recovery, during which animals are more susceptible to disease as well as other environmental stress. With a lack of available energy stores, post-spawning animals lack the ability to cope with other stressors, thus spawning in the commercial stock creates an increased risk of stress and

mortality. Therefore, being able to predict when sexual maturation occurs or when abalone prepare to spawn can reduce the mortality due to the stress of spawning and produce higher quality animals. Continuing research in this area is pivotal and has already began (Botwright *et al.*, 2014)

Spawning and decreased disease resistance is one example of the synergistic effects of multiple stresses. In general, most research has focussed on the effects of a single stress event to understand how abalone cope with certain stressors. Now is the time where it is needed to start producing research on combined effects of various stresses. The farm environment is a dynamic one, and it is unlikely to find individual stressors. Abalone have a host of defence mechanisms against most environmental and farm stresses, and when we start to investigate the additive effects we will begin to understand the true environment in which they are living. Furthermore, the recovery times from additive stressors are likely to be much longer than what we believe it to be for a single stress. Answering these types of questions will allow farmers to adapt their protocols and prevent mass mortalities, produce higher quality animals and become more profitable. In the same respect, the industry will not only need to focus on prevention and recovery from stress, but prevention and recovery from disease. There is a wide array of bacteria and viruses that can infect abalone and disease prevalence becomes higher on farms where stocking density is high. Future research on the ecology of these diseases will enable better prevention and yield new targets for selective breeding against resistance and/or new therapies to destroy the infectious agents.

Once we understand the mechanisms behind the effects of these stresses we can then move on to interventions. Aside from the aforementioned investments in infrastructure, the next most obvious vehicle for interventions is manipulations of the diet and/or genetics, and this research is already underway to help combat thermal stress. The feed represents a mechanism by which small molecules can be delivered to prevent maturation, combat ROS production during hypoxia, and protect against osmoregulatory or ionic (ammonia and/or pH) stresses, for example. The diet can also be used to alter the energetic status of the animals which could potentially help to mitigate the effects of stress on energy reserves.

Now is a crucial time where understanding the mechanistic physiology of abalone would give an advantage as the climate continues to change, and farming practices become more industrialised. If a clear understanding of the effects of environmental stress and how these effects interact with farming procedures is obtained than it can be predicted how best to modify farming practices to ensure the abalone industry remains sustainable.

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CHAPTER 3: Respiratory response of early life abalone

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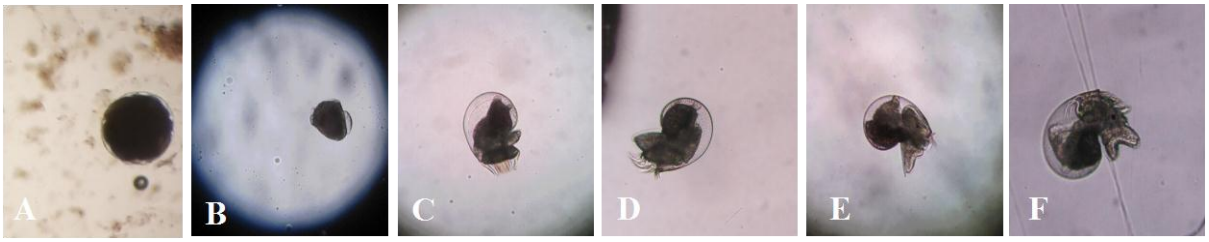

Early-life stages of hybrid abalone examined in this chapter: Fertilised egg (A), trochophore larva (B), early veliger larva (C), late veliger larvae (D, E) and settling larva (F).

Hatchery conditions do not negatively impact respiratory response of early life-stage development in Australian hybrid abalone

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Abstract

On Australian aquaculture farms, early life stages of abalone are reared under controlled abiotic and biotic conditions in an attempt to optimise individual growth and reduce potential stressors. Yet, physiological responses to the rearing conditions are largely unknown. This study tests if commercial stocking densities, light conditions and oxygen levels influence the oxygen consumption rate ($\dot{M}O_2$) of early life stages of *H. rubra* and *H. laevigata* hybrids at a standard commercial hatchery temperature of 16 °C. Oxygen consumption rate of fertilised eggs and larvae in the trochophore, mid veliger and early benthic veliger stages were measured at densities from 100 to 2400 individuals ml^{-1}, in light and dark conditions and oxygen levels of 100 to 0% O_2sat. Neither density nor light conditions affected $\dot{M}O_2$ of any of the tested life stages. Normoxic $\dot{M}O_2$ varied across developmental stage and was higher in the actively swimming mid veliger stages (114.92 ± 2.68 pmol O_2 ind^{-1} h^{-1}) in comparison to less active earlier (49.48 ± 2.33 pmol O_2 ind^{-1} h^{-1}) and later life stages (65.90 ± 3.05 pmol O_2 ind^{-1} h^{-1}). Critical oxygen tensions, taken as the point at which animals could no longer maintain $\dot{M}O_2$, ranged from 22.7 ± 2.7% O_2sat in fertilised eggs to 14.0 ± 1.0% O_2sat in mid veliger larvae. These results suggest that current conditions in Australian abalone aquaculture farms should not negatively impact the development of early life stages of hybrid abalone.

Key words: hybrids, hatchery, metabolism, ontogeny, respiration

Introduction

Aquaculture industries attempt to optimise farm conditions to maximise growth and survival of their stock. During land-based cultivation in Australia, early life stages of abalone are reared under relatively well-controlled hatchery conditions. Stocking densities are managed and temperature, oxygen levels (to some extent), and photoperiod are controlled in an effort to minimise stressors during this critical hatchery phase (Heasman and Savva, 2007). This is important as growth of an individual is influenced by the abiotic conditions it is exposed to (Webber and Riordan, 1976; Sebens, 1987), and survival and healthy recruitment into the nursery are key objectives for abalone farms because early life growth influences juvenile performance (Daume *et al.*, 2004) and ultimately promotes sustainability.

The physiological responses of early life-stage abalone to rearing conditions including density, oxygen availability, and light levels are largely unknown. On Australian aquaculture farms, protocols suggest fertilised eggs and larvae are stocked at approximately 20 individuals ml^{-1} (Heasman and Savva, 2007). This density might not influence the physiology of early life stages as it has been shown that densities of 65 to 900 larvae ml^{-1} have no influence on oxygen consumption rates ($\dot{M}O_2$) of *H. fulgens* Philippi and *H. rufescens* (Shilling *et al.*, 1996; Moran and Manahan, 2003). Yet, density effects on fertilised eggs of *Haliotis* species remain unknown. Oxygen is among the key abiotic factors controlling species performance, as it is a necessary electron acceptor of the aerobic energy pathway. Below the critical oxygen tension (P_{crit}), $\dot{M}O_2$ drops rapidly, and animals increasingly rely on the less energy efficient anaerobic metabolism (Wieser, 1986; Pörtner and Grieshaber, 1993). Despite its importance, oxygen saturation (% O_2sat) in the rearing tanks is not monitored, but a low airflow is provided (L. J. McPherson, Jade Tiger Abalone (JTA), pers. comm., November 2014). Further, the P_{crit} of fertilised eggs and larvae of *Haliotis* is unknown and thus, it is unclear whether current rearing conditions enable larvae to maintain aerobic metabolism and grow optimally. In Australian abalone hatcheries, lights are mostly switched off as it has been observed that under these conditions larvae spread more evenly throughout the water column (L. J. McPherson, JTA, pers. comm., November 2014). This may be an indication for positive phototaxis, which in turn may indicate that light conditions cause an increase in $\dot{M}O_2$, as seen in older abalone (Ahmed *et al.*, 2008a). The only comparable study at young life stages showed that light intensities had no influence on survival rates of *H. asinina* (Madrones-Ladja and Polohan, 2001).

In Australia, hybrid abalone of *H. rubra* and *H. laevigata* are produced in selective breeding and hybridization programmes. In general, applied quantitative genetics are deployed to produce a product that has desired characteristics, such as improved growth rates or higher tolerances to abiotic factors (Leighton and Lewis, 1982; Elliott, 2000; Cheng *et al*., 2006; Kube *et al*., 2007; Hamilton *et al*., 2009). The objective of this study was to assess early life aerobic energy requirements of the hybrid of *H. rubra* and *H. laevigata* abalone under a range of abiotic and biotic conditions relevant to hatchery activities. Biotic parameters included life stage and density of individuals per respiration chamber. Abiotic factors comprised oxygen levels (% O_2sat) and light condition. It was hypothesised that $\dot{M}O_2$ is enhanced in actively swimming life stages at lower densities, at high O_2sat, and in light conditions.

Methods

Animal rearing

Broodstock conditioning, spawning, fertilization, and rearing of early life stages of *H. rubra* and *H. laevigata* hybrids were performed according to standard farm practices at the aquaculture farm JTA, Indented Head, Victoria, Australia, in March 2015. Broodstock were from the JTA selective breeding programme and had been selected on the basis of their hybrid performance using hybrid breeding values. Females were *H. rubra* from the 2010 y class (4.5-y-old), and males were *H. laevigata* from the 2011 y class (3.5-y-old). Females and males were two and three generations from founder (wild) stock, respectively. All parents had been selected for the same breeding objective, improved growth, and were representative of the best performers in the population. Offspring from four families (P1–P4) were used for experiments and tested separately to reduce genetic variance influencing the results. The individuals within families were related as half-sib families, with a single mother and five fathers.

In the rearing tanks, a constant low flow of air was provided to the low flow of filtered seawater (1 μm, ultraviolet treated) at 16 °C. Animals were transferred to new tanks first 2 days after fertilization and subsequently every other day.

Experimental design

The $\dot{M}O_2$ of early life-stage abalone hybrids was measured at 1, 25, 56, 74, 97, and 146 h postfertilization (hpf; replicate per stage = 8 to 13) with a randomly chosen number of individuals per replicate so that densities of individuals per respiration chamber intentionally

varied within and between life stages (Table 3.1). Subsamples were taken at the commencement of each experiment to determine developmental stage based on morphological characteristics observed under a microscope (Fig. 3.1, Table 3.1; Hone *et al*., 1997). Oxygen consumption rate of offspring from family P1 was measured at 1, 25, 56, 74, and 146 hpf. Offspring from family P2 were tested at 1, 74, and 146 hpf and offspring from families P3 and P4 were used for experiments at 97 hpf. The major study group, offspring from family P1, was tested at five developmental stages to gain a broad insight into the respiratory response across development. Offspring from families P2–P4 were used to test for genetic variance and were used for experiments at fewer developmental stages because of the fast developmental rate of early-life stages of abalone.

Oxygen consumption rate measurements were conducted in a temperature-controlled room at 16 °C. Lights were switched on during experimental runs, and half of the respiration chambers were covered with aluminium foil to test for effects of light condition on $\dot{M}O_2$. Measurements were conducted during the day except for 56 hpf individuals. These were used for experiments during the night.

Respiration chambers consisted of galvanised aluminium plates with 24 wells (2.7 ml). Percentage O_2sat was measured with a sensor dish reader (PreSens, Germany) throughout the experimental period at a time interval of 15 s. Individuals were transferred to chambers filled with supersaturated seawater (maximum 120% O_2sat) to ensure normoxic conditions when experimental runs commenced. Measurement of $\dot{M}O_2$ started immediately after all chambers were hermetically sealed with individual acrylic lids. Measurements were terminated when O_2sat dropped to 80% for experiments with offspring from families P2–P4. To determine P_{crit}, the point when individuals change from oxyregulators to oxyconformers, $\dot{M}O_2$ measurements with offspring from family P1 were terminated when % O_2sat reached 10% to 0% O_2sat. Two-point calibrations were performed in air-saturated seawater for 100% O_2sat and in sodium sulphite–saturated seawater for 0% O_2sat. For each life stage, four chambers without individuals served as blanks to account for microbial respiration. After experiments, individuals were rinsed with fresh water, preserved in 10% formalin, and transported to CSIRO, Hobart, Tasmania. Individuals were counted and SL was measured (Table 3.1).

Statistical analyses

Critical oxygen tension of family P1 offspring was calculated by means of the smallest sum of the residual sum of squares of two linear regressions fitted to individual $\dot{M}O_2$ data using R

(Yeager and Ultsch, 1989). One-way analysis of covariance was conducted for each life stage to test for the effect of density, light condition, family, and P_{crit} on $\dot{M}O_2$. Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks was conducted to determine the effect of life stage on $\dot{M}O_2$ within families P1 and P2. Normality was tested with Shapiro–Wilk tests, and homogeneity of variance was tested with Brown–Forsythe tests. Multiple pairwise comparisons were conducted with Dunn's method. Probability of less than 0.05 was considered for rejecting the null hypothesis.

Table 3.1: Development, size, and tested density of *Haliotis rubra* × *H. laevigata* hybrid abalone during course of experiment.

Age*	Characteristics	Life stage	Family	Average size (µm)	Size range (µm)	Density (ind ml^{-1})
1	Gastrula	Fertilised eggs	P1	226 ± 1	205–245	1298–2341
			P2	219 ± 2	187–226	888–2228
25	Prototrochal grindle larval shell rudiments	Trochophore larvae	P1	261 ± 2	234–284	642–1468
56	Velum food mass	Mid-veliger	P1	281 ± 1	261–305	178–767
74	integumental attachment with larval shell	Mid-veliger	P1	280 ± 1	261–296	103–948
			P2	276 ± 1	256–306	219–811
97	Eye spots	Late veliger	P3	280 ± 1	259–294	257–751
			P4	284 ± 2	273–295	278–621
146	Cilia on roof of mantle cavity tentacles	Early benthic veliger	P1	280 ± 2	265–294	333–932
			P2	284 ± 1	255–305	217–917

Average size values are mean ± SE.
* = Age, in hpf, states the time when individuals were transferred to respiration chambers.

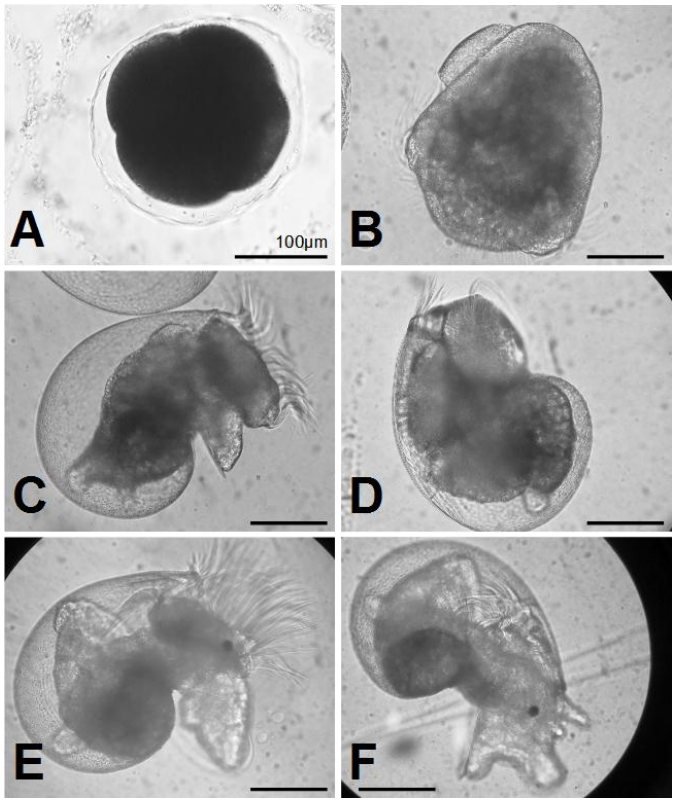

Fig. 3.1: Development of individuals from family P1. (A) 1 hpf (fertilised eggs), (B) 25 hpf (trochophore stage larvae), (C) 56 hpf and (D) 74 hpf (mid-veliger stage larvae), (E) 97 hpf (late veliger stage larvae from parents P3), and (F) 146 hpf (veliger larvae in early benthic stage). Scale = 100 µm.

Results

Density and light condition

For all life stages, $\dot{M}O_2$ was unaffected by density ($p > 0.05$; Fig. 3.2). Individuals at 1 and 25 hpf were tested up to densities of 2341 and 1468 ind ml^{-1}, respectively, whereas later life stages were only tested with a maximum of 948 ind ml^{-1} (Table 3.1). Light condition had no effect on $\dot{M}O_2$ of tested life stages ($p > 0.05$; Fig. 3.2). Hence, data for each life stage were pooled and used to test for differences in $\dot{M}O_2$ between life stages and families.

Life stages

For offspring of family P1, one-way ANOVA showed differences in $\dot{M}O_2$ between life stages of the hybrid abalone ($p < 0.001$; Fig. 3.3A). Individuals at 56 hpf (120.79 ± 4.33 pmol O_2 ind^{-1} h^{-1}) and 74 hpf (109.95 ± 2.79 pmol O_2 ind^{-1} h^{-1}) had significantly higher $\dot{M}O_2$ compared with earlier (1 hpf: 46.67 ± 3.29 pmol O_2 ind^{-1} h^{-1}; 25 hpf: 52.47 ± 2.72 pmol O_2 ind^{-1} h^{-1}) and later (146 hpf: 65.90 ± 3.05 pmol O_2 ind^{-1} h^{-1}) life stages (Fig. 3.3A, Table 3.2). Individuals from P2 showed a similar pattern, where $\dot{M}O_2$ increased from 1 hpf (43.74 ± 3.37 pmol O_2 ind^{-1} h^{-1}) to 74 hpf (146.15 ± 4.22 pmol O_2 ind^{-1} h^{-1}) and decreased again from 74 to 146 hpf (97.68 ± 2.42 pmol O_2 ind^{-1} h^{-1}; $p > 0.05$) (Fig. 3.3B, Table 3.2). At 97 hpf, $\dot{M}O_2$ of offspring from family P3 (103.91 ± 4.48 pmol O_2 ind^{-1} h^{-1}) and P4 (99.14 ± 4.05 pmol O_2 ind^{-1} h^{-1}) was in between $\dot{M}O_2$ values of 56 and 146 hpf originating from both families P1 and P2 (Fig. 3.3B, Table 3.2).

Family influence

At 1 hpf, average $\dot{M}O_2$ of individuals from families P1 and P2 was similar ($p > 0.05$; Fig. 3.3). At 74 and 146 hpf, $\dot{M}O_2$ of individuals from family P2 was significantly higher compared with $\dot{M}O_2$ of individuals of family P1 ($p < 0.05$; Fig. 3.3). Values of $\dot{M}O_2$ of 97 hpf individuals were similar between families P3 and P4 ($p > 0.05$; Fig. 3.3B).

Oxygen level

Percent oxygen saturation influenced $\dot{M}O_2$ of all life stages similarly in that individuals were able to regulate oxygen uptake over wide oxygen ranges ($p > 0.05$; Fig. 3.4). Average P_{crit} values were independent of density and ranged from 22.7 ± 2.7% O_2sat in 1 hpf individuals to 14.0 ± 1.0% O_2sat in 74 hpf individuals.

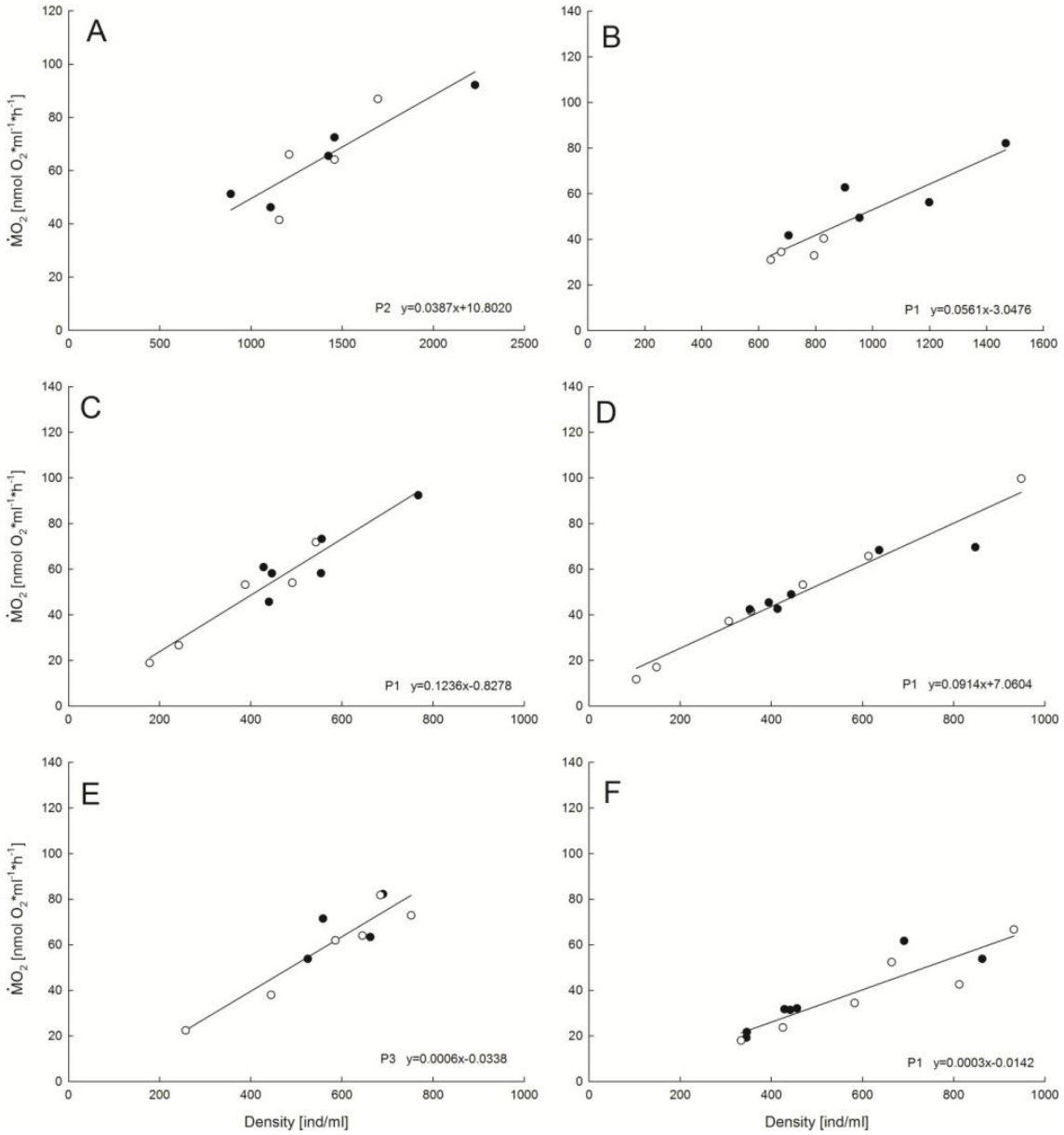

Fig. 3.2: Oxygen consumption rate (nmol O_2 ml^{-1} h^{-1}) of (A) 1-, (B) 25-, (C) 56-, (D) 74-, (E) 97-, and (F) 146-hpf-old abalone hybrids at various densities (ind ml^{-1}) originating from families P1 (25, 56, 74, and 146 hpf), P2 (1 hpf), and P3 (97 hpf). Open symbols represent light and closed symbols represent dark conditions.

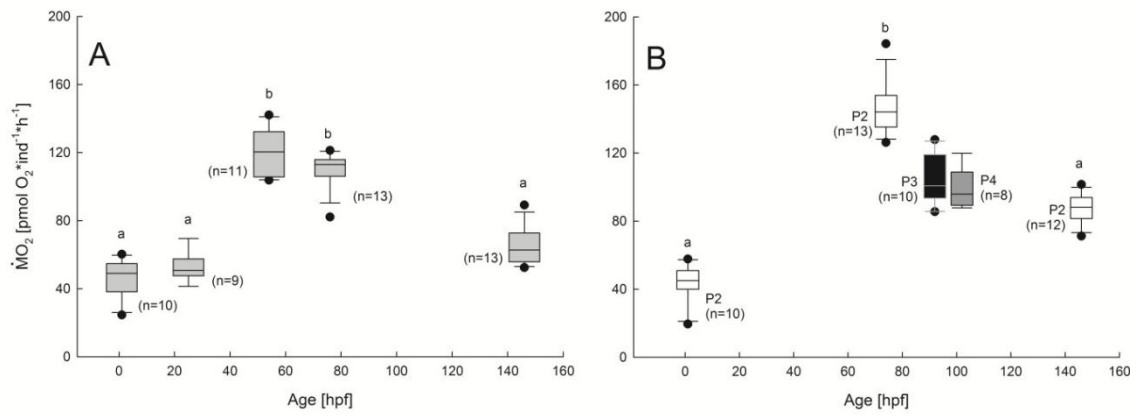

Fig. 3.3: Effect of age (hpf) on $\dot{M}O_2$ (pmol O_2 ind^{-1} h^{-1}) in offspring from (A) family P1 and (B) family P2 (open), family P3 (dark shaded), and family P4 (light shaded). Lowercase letters indicate significantly different $\dot{M}O_2$ among life stages within P1 and P2 (one-way ANOVA on ranks, Dunn's method, $p < 0.05$). The box shows the 95% range with the median. Whiskers represent lowest and highest values.

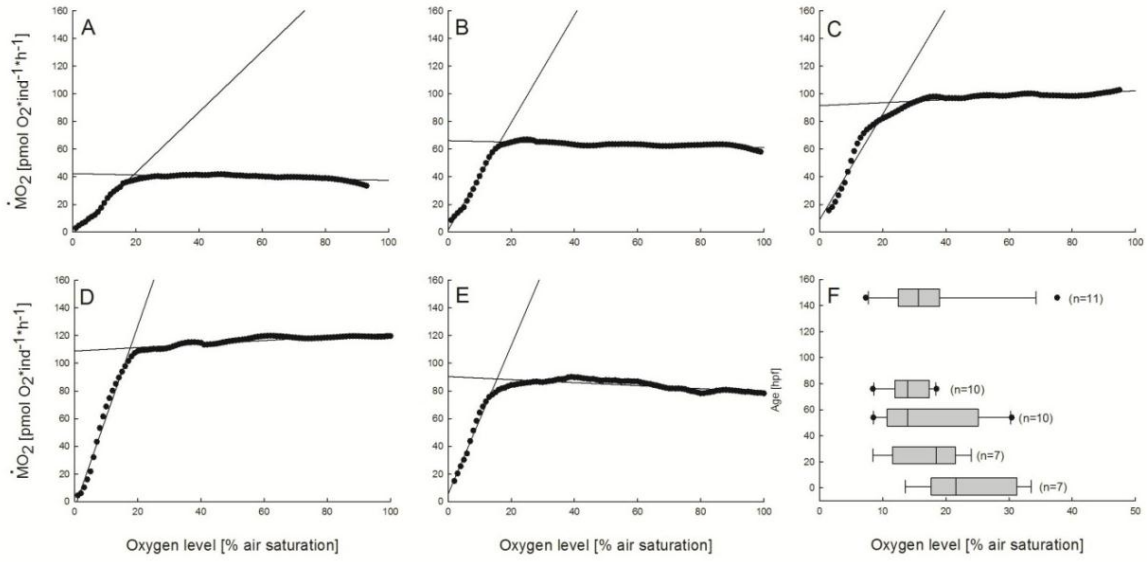

Fig. 3.4: Effect of oxygen level (% O_2sat) on $\dot{M}O_2$ (pmol O_2 ind^{-1} h^{-1}) on (A) 1, (B) 25, (C) 56, (D) 74, and (E) 146 hpf for hybrid family P1. Each panel (A–E) shows a representative individual within one developmental stage. The intersection of the two regression slopes shows the P_{crit} value, the point where individuals change from oxygen regulators to conformers. (F) The box plot shows mean and range P_{crit} values at each of the five life stages examined. The box shows the 95% range with the median. Whiskers represent lowest and highest values.

Table 3.2: Comparison of $\dot{M}O_2$ (pmol O_2 ind^{-1} h^{-1}) of fertilised eggs and larvae of *Haliotis* species during development stated in literature and present study.

	Type of abalone									
	H. rufescens				***H. fulgens***		***H. rubra* × *H. laevigata* hybrid**			
Family/culture	C E	C C	C G	C1	C1	C2	P1	P2	P3	P4
Developmental stage										
Embryo	–	–	–	–	–	–	47	44	–	–
Preveliger	–	–	–	~50	~65	~40	53	–	–	–
Early veliger	–	307	347	~140	~90	~75	121 (56*)	–	–	–
							110 (74*)	146 (74*)	–	–
Late veliger	197	302	288	~90	~75	~95	–	–	104 (97*)	99 (97*)
Settling larvae	139	222	251	–	~80	~80	66	98	–	–
Early juveniles	259–773	705–1659	425–911	–	–	–	–	–	–	–
Temperature	13	15	15	17	15	15	16	16	16	16
Reference	Shilling *et al.* (1996)			Jaeckle and Manahan (1989)	Moran and Manahan (2003)		Present study			

C = different cultures; P = families in this study
* = Age in hours post fertilization.

Discussion

Oxygen consumption rates of early life-stage *H. rubra* × *H. laevigata* hybrids were dependent on developmental stage and likely attributable to differences in activity levels. At 1 hpf, individuals were at the inactive gastrula stage and had the lowest $\dot{M}O_2$ values in comparison with later life stages. The major tissue of fertilised eggs is storage material (Crofts, 1937), particularly for the lecithotrophic abalone development, and this corresponds with the low $\dot{M}O_2$ reported in this study. At 25 hpf, individuals had developed to trochophore larvae that were actively swimming using cilial movement of their velum. Yet, $\dot{M}O_2$ values remained low, possibly because of limited muscular activity as the cardiovascular system and muscles are not yet developed (Crofts, 1937). Oxygen consumption rate significantly increased in 56- and 74-hpf-old individuals. At this mid-veliger larvae stage, rudiments of cardiovascular systems and ventilation organs have developed that will continue to differentiate until individuals develop into postveligers (Crofts, 1937; Shilling *et al.*, 1996). Further, retractor muscles have developed and frequent muscle contractions are likely to be largely responsible for higher $\dot{M}O_2$. In laboratory conditions, constant swimming of pelagic larvae was only

interrupted for short periods when individuals swam into each other or into the tank wall (Wong *et al*., 2010; pers. obs. from this study). The larvae respond to this impact by retracting the body into the shell, but quickly return to swimming. In contrast, later stage larvae in the early benthic life stage form can rest as they start to settle, allowing metabolic rates to decrease compared with periods of higher locomotor activity. At this later stage, they periodically attach to surfaces and start swimming again only if the substrate is unsuitable for settlement (Moss and Tong, 1992; Mullineaux and Garland, 1993; Najmudeen and Victor, 2004). In accordance, 146 hpf individuals in this study were at the early benthic life stage, and $\dot{M}O_2$ was decreased in comparison with mid-veliger stages (Fig. 3.3).

The resulting pattern of a low $\dot{M}O_2$ in fertilised eggs and trochophore larvae, a peak in $\dot{M}O_2$ of mid-veliger larvae, and a subsequent drop in $\dot{M}O_2$ in early benthic larvae was independent of family (Fig. 3.3). For individuals from family P1, mean normoxic $\dot{M}O_2$ of fertilised eggs was 40% and 65% of $\dot{M}O_2$ of mid-veligers (56 to 74 hpf) and early benthic veligers (146 hpf), respectively. Oxygen consumption rate of fertilised eggs from family P2 was 30% and 55% of that of mid-veligers (56 hpf) and early benthic veligers (146 hpf), respectively. A similar trend for $\dot{M}O_2$ across larval development has also been reported for early life stages of *H. fulgens* (Moran and Manahan, 2003). In *H. fulgens*, $\dot{M}O_2$ increased gradually from preveligers to early veligers and late veligers. Oxygen consumption rate of settling larvae had decreased back to levels similar to those of early veligers (Table 3.2; Moran and Manahan, 2003). Two further studies that investigated energy requirements of *H. rufescens* did not discuss dropping $\dot{M}O_2$ from early veligers to late veligers (Jaeckle and Manahan, 1989; Table 3.2) and from late veliger to settling larvae (Shilling *et al*., 1996; Table 3.2).

Oxygen consumption rate of fertilised eggs and larvae was influenced by family. Although at 97 hpf, $\dot{M}O_2$ was similar in larvae from family P3 and P4 (Fig. 3.3), at 74 and 146 hpf, $\dot{M}O_2$ of larvae originating from families P1 and P2 was different in that $\dot{M}O_2$ of offspring from P2 was higher. Also Moran and Manahan (2003) calculated $\dot{M}O_2$ of early life stages of *H. fulgens* originating from two sets of parents and found differences in $\dot{M}O_2$ values at similar life stages. Larval performance is likely to be strongly influenced by heritable (genetic) and maternal (egg quality) factors. The "compensation hypothesis" presumes that individuals possess higher growth potential because of a lower standard metabolic rate as the reduced energy costs for the maintenance of baseline metabolism can be channelled into growth (reviewed by Burton *et al*. (2011)). Conversely, the "increased intake hypothesis" suggests that individuals with higher growth rate potential possess a higher standard metabolic rate

and also an increased maximum metabolic rate, this enables the individual to gain more energy per time unit, thus resulting in improved growth rates (reviewed by Burton *et al.* (2011)). It has been suggested that the latter hypothesis might only be valid for environments with an excess supply of food, such as in aquaculture facilities and during lecithotrophic development (reviewed by Burton *et al.* (2011)). Yet, a large-scale study regarding $\dot{M}O_2$ of early life stages of abalone originating from different families is needed to reveal if a relationship between $\dot{M}O_2$ of offspring and growth rates and maternal influences of parents exists and if selective breeding and/or broodstock conditioning influences $\dot{M}O_2$ in the earliest life stages.

The metabolic response to % O_2sat was independent of life stage in this study, and $\dot{M}O_2$ was reduced only at O_2sat levels lower than 20% O_2sat, well below what is expected in a hatchery. Early life stages of abalone take up oxygen mainly by diffusion that is enhanced by movement of cilia to increase water flow over the body. It might be that the energy required for cilial movement is not met under continuously low % O_2sat, resulting in lower diffusion rates and thus lower $\dot{M}O_2$. Similarly, a reduction in swimming velocity below the P_{crit} has been reported for other gastropod species. Swimming velocity of the larvae of *Nassarius siquijorensis* A. Adams and *N. conoidalis* Deshayes was influenced at 34% O_2sat in 10-day-old *N. siquijorensis* and at 14% O_2sat (calculated with 24 °C, salinity 30, and 1,000 hPa) in *N. cornoidalis* (Liu *et al.*, 2011). Yet, behaviour of early life stages was not observed during experiments in this study and warrant further investigation.

To our knowledge, this is the first study to report on hypoxia tolerance across full larval development from fertilised eggs to near settling larvae of *Haliotis*. Yet, $\dot{M}O_2$ of other invertebrate larvae, such as the echinoid *Dendraster excentricus* Eschscholtz, the asteroid *Asterina miniata* (Brandt), and the decapod *Petrolisthes laevigatus* Guérin, were also stable until O_2sat dropped below 20% (Hoegh-Guldberg and Manahan, 1995; Alter *et al.*, 2015). Oxygen sensitivity may change across wider temporal scales. Although the fertilised eggs and larvae in this study were able to maintain $\dot{M}O_2$ over a wider oxygen range, 4-mo-old *H. rubra* × *H. laevigata* hybrids were only able to regulate $\dot{M}O_2$ until 40% O_2sat (K. Alter, unpublished data), and 4-y-old *H. laevigata* have been reported to have poor hypoxia tolerance with a P_{crit} of only 80% O_2sat (Harris *et al.*, 1999).

Relationships between $\dot{M}O_2$ and density were linear up to the highest tested densities, indicating that $\dot{M}O_2$ of all tested life stages was unaffected by the experimental conditions. In this study, life stages were tested with high numbers of individuals per respiration chamber.

Fertilised eggs (1 hpf) were tested with densities between 888 and 2340 ind ml^{-1}, and early benthic veligers were tested with densities between 333 and 932 ind ml^{-1} (Table 3.1). In accordance, previous studies reported that $\dot{M}O_2$ is not influenced up to the minimum densities tested in this study. Oxygen consumption rate of veligers and early post-settlement individuals of *H. rufescens* was not affected by densities from 22 to 911 ind ml^{-1} and 44 to 243 ind ml^{-1}, respectively (Shilling *et al*., 1996). Oxygen consumption rate of early life stages of other marine invertebrates has been determined at densities with a range of 241 to 809 ind ml^{-1} for larvae of oyster *Crassostrea gigas* (Thunberg) and 16 to 669 ind ml^{-1} for embryos of sea urchin *Strongylocentrotus purpuratus* Stimpson, with no effect of density (Hoegh-Guldberg and Manahan, 1995; Marsh and Manahan, 1999).

No difference in $\dot{M}O_2$ was detected when individuals were exposed to either light or dark conditions. During $\dot{M}O_2$ measurements of early life stages in this study, respiration chambers were either placed under a light source or covered with foil to create lighter and darker conditions, respectively. Yet, $\dot{M}O_2$ was measured with an optical oxygen sensor that emitted a blue light every 15 s during experimental periods, including dark conditions. If there is an effect of light on $\dot{M}O_2$, it might be that the continuous flashing of blue light prevented the detection. Shortly after hatching, trochophore larvae start swimming toward the water surface where they remain until settlement. It has been suggested that this behaviour is caused by positive phototaxis, yet some other studies proposed negative geotaxis (Yano and Ogawa, 1977; Sasaki and Shepherd, 1995; Leighton, 1989). If pelagic larvae are positively phototactic and become negatively phototactic during the settlement stage it is likely that light conditions would have had an influence on $\dot{M}O_2$, as has been shown for juvenile individuals (Ahmed *et al*., 2008a). Oxygen consumption rate of juveniles of *H. discus discus*, *H. gigantea* Gmelin, *H. madaka* Habe, and their interspecies hybrids increased when exposed to light conditions compared with exposure to dark conditions (Ahmed *et al*., 2008a).

In summary, early life abalone in Australian aquaculture are reared under controlled conditions, such as 16 °C, high oxygen levels, predominantly dark conditions, and densities of 20 ind ml^{-1} (Heasman and Savva, 2007; L. J. McPherson, JTA, pers. comm., November 2014). In this study, $\dot{M}O_2$ of fertilised eggs, trochophore larvae, mid and late veligers as well as early benthic veligers of *H. rubra* × *H. laevigata* hybrids was unaffected by densities of up to 2400 ind ml^{-1}. Further, no difference in $\dot{M}O_2$ was detected when individuals were exposed to lighter or darker conditions, and $\dot{M}O_2$ of all tested life stages was reduced only when oxygen levels dropped below 20% O_2sat. These results suggest that the typical biotic and

abiotic conditions in abalone aquaculture should not hamper development of early life hybrids.

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CHAPTER 4: Thermal preference of abalone larvae

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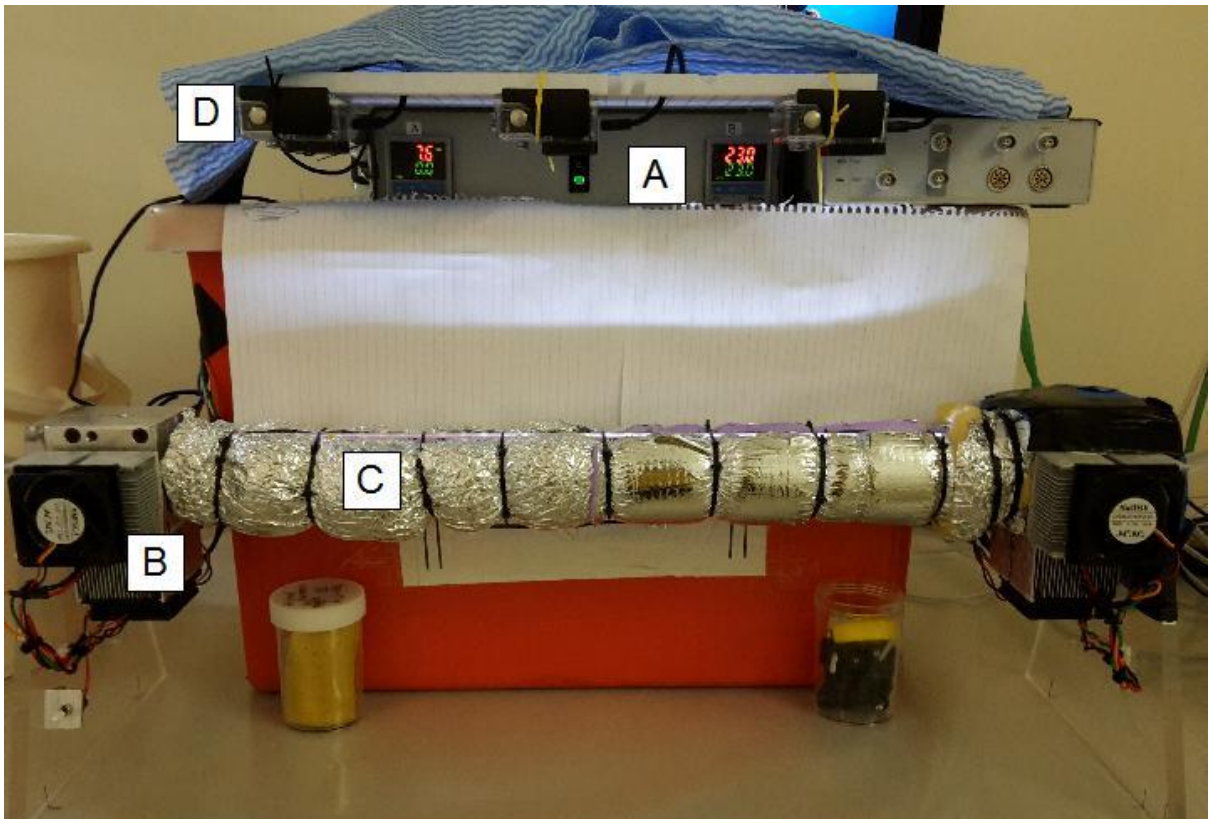

The horizontal thermal gradient apparatus used in this chapter consisted of a custom built electronic controller (A) from the University of Tasmania that maintained Peltier devices (B) attached at both ends of the gradient (C). The gradient comprised a glass tube that was embedded in an aluminium block and insulated with foam. A slot in the aluminium block across the whole length of the glass tube served as a viewing window for three cameras that were installed above (D).

Thermal preference increases during larval development of pure and hybrid abalone

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Abstract

Temperature is one of the main factors influencing biological processes of ectothermic species. An optimum temperature range of 16 to 18 °C has been suggested for the development of early life stages of temperate Australian abalone, yet there are little physiological or behavioural data to support this suggestion. This study examines the acute thermal preferences (T_{pref}), swimming speeds (*U*) and oxygen consumption rates ($\dot{M}O_2$) of veliger larvae of blacklip abalone (*H. rubra*), greenlip abalone (*H. laevigata*) and their interspecies hybrid. Thermal preference and *U* were measured in a thermal gradient with temperatures ranging from 12 to 25 °C, and $\dot{M}O_2$ was measured at 4 to 7 temperatures between 12 and 32 °C. Thermal preference increased in all three groups of abalone during development from a T_{pref} of 16 °C in one day-old early veligers to a T_{pref} of 20 °C in three day-old late veligers. Swimming speed increased with temperature in all three groups of abalone and increased with age in *H. rubra* and hybrids but not in *H. laevigata*. Veliger $\dot{M}O_2$ increased throughout the ecologically relevant temperature range in all three abalone groups. Higher temperatures were examined in hybrids, and it was found that $\dot{M}O_2$ reached a peak at 25 °C and declined thereafter. These results align with the temperatures that veligers may experience in their natural habitat and provide support that current temperatures maintained at Australian aquaculture hatcheries are within optimal ranges for larval performance.

Keywords: Behavioural thermoregulation, metabolism, early-life stages, abalone

Introduction

Temperature is a major abiotic factor influencing biological functions of ectotherms (Angilletta, 2009). Through behavioural thermoregulation, ectothermic organisms can optimise performance traits to enhance survival and fitness. For example, temperatures can be selected to optimise digestive efficiency to enhance growth rates, or to optimise swimming speed to reduce the risk of predation (Videler, 1993; Green and Fisher, 2004; Chapperon and Seuront, 2011a; Chapperon and Seuront, 2011b). Studies of behavioural thermoregulation in marine gastropods have been conducted on adults (Casterlin and Reynolds, 1980; Hecht, 1994; Muñoz *et al*., 2005), however, so far no study has examined the larval life stage. Assessing thermal preferences and tolerances of larvae can give insight into dispersal ranges and species distributions because they often reflect the thermal experiences encountered in the natural habitat (Hammond and Hofmann, 2010; Zippay and Hofmann, 2010).

Larvae of the blacklip abalone *H. rubra*, greenlip abalone *H. laevigata* and their interspecies hybrid are potentially exposed to temperatures similar to those of adult individuals, because it has been suggested that larvae have a low dispersal range and develop in habitats where adults reside (Prince *et al*., 1987). Nevertheless, this idea remains speculative, with only a few larvae of *H. rubra* and no larvae of *H. laevigata* or their hybrid having been collected in the wild (Prince *et al*., 1987; Babcock and Keesing, 1999). Both pure species occur along the southern coast of Australia, including Tasmania. The interspecies hybrid is rare in nature, although adult hybrids have been found in areas where the two pure species live sympatrically (Brown, 1995). Adults of *H. rubra* inhabit sheltered areas with rocks and caves. Their distribution range extends further south than that of *H. laevigata* so that *H. rubra* experiences slightly colder temperatures throughout the year and is most commonly found at temperatures between 11 and 19 °C. Adults of *H. laevigata* occur on rocks in open sandy areas, where the most common habitat temperatures range between 12 and 23 °C throughout the year (Shepherd, 1973).

The hybrid is becoming a key commercial asset in Australian aquaculture because of its apparent growth advantage in the juvenile stage, yet the two pure species are also commercially cultured in Australia (Guo, 2009). Current best industry practice recommends that embryos and larvae of *H. rubra* should be reared at 16 to 18 °C (Heasman and Savva, 2007). This temperature recommendation has also been adopted by aquaculture farmers for the rearing of *H. laevigata* and hybrid larvae (L. McPherson, Jade Tiger Abalone, pers. com. November 2014). Upper critical temperatures of the lecitotrophic larvae have not been

reported but it has been shown that larval development slows at temperatures lower than 16 °C and is arrested completely at 7.8 °C in *H. rubra* and 7.2 °C in *H. laevigata* (Grubert and Ritar, 2004). While this general guideline for aquaculture (rearing at 16 to 18 °C) has been established based on growth and survival, little is known about the physiological and behavioural responses of *Haliotis* larvae to temperature. To address this knowledge gap, this study quantifies acute thermal preferences (T_{pref}), swimming speeds (*U*) and oxygen consumption rates ($\dot{M}O_2$) of larval veliger *H. rubra*, *H. laevigata* and their hybrid across an ecologically relevant temperature range. Moreover, given its significant commercial importance, the hybrid was challenged with higher temperatures to understand upper thermal tolerance limits. It was hypothesised that T_{pref} for larvae would be highest for *H. laevigata* and lowest for *H. rubra* based on the distribution of adult abalone and their natural habitat temperatures (Shepherd, 1973). Further, it was predicted that upper thermal limits of hybrids would be marked by a decline in $\dot{M}O_2$ once naturally-occurring temperatures were exceeded.

Methods

Animal rearing

Larvae from *H. laevigata*, *H. rubra* and their interspecies hybrid (*H. rubra* female by *H. laevigata* male) were reared according to standard farm practices at the Jade Tiger Abalone (JTA) hatchery in Indented Head, Australia. All experiments were conducted at the hatchery. Larvae were initially held in constantly flowing filtered sea water (filtered to 1 micron, UV-treated), at 16.8 ± 0.1 °C and 100% O_2sat. Hybrid, *H. laevigata* and *H. rubra* larvae were obtained from three commercial spawning events in November 2015, December 2015 and March 2016. Multiple spawning events were required to obtain a large enough sample size due to varying fertilization success rates and logistical constraints on data collection from larvae which develop quickly and only spend ~ 3 days at 17 °C in the target veliger form.

Oxygen consumption rate of hybrids is dependent on developmental stage and it has been suggested that differences in $\dot{M}O_2$ across the different stages (embryonic, trochophore larvae, veliger larvae and settling larvae) are attributable to differences in activity level (Chapter 3). Thus, only veliger larvae were chosen in the present study to target a developmental stage at which the mode of locomotion is stable. The first, second and third day of the veliger larvae stage correspond to effective accumulative temperatures (EAT) of 394 to 553 °C-h, 624 to 788 °C-h, and 842 to 1008 °C-h, respectively (Table 4.1 and 4.2).

Table 4.1: Experimental temperature (Temperature) [°C], developmental day and corresponding effective accumulative temperature (EAT) [°C-h], number of individuals [ind ml^{-1}], number of replicates and shell length (average size [µm] mean ± SE, size range [µm]) of *H. rubra*, *H. laevigata* and hybrids from the December populations and hybrids from the March population used for oxygen consumption measurements.

Date	**Species**	**Temperature**	**Development**		**Individuals**	**Replicates**	**Size**	
			Veliger day	**EAT**		***n***	**Mean ± SE**	**Range**
		[°C]		**[°C-h]**	**[ind ml^{-1}]**		**[µm]**	**[µm]**
Dec-15	*H. rubra*	12	1	464	171- 460	7	294±3	270-309
		17	1	519	77- 650	7	291±3	277-308
		17	2	655	293- 554	5	294±2	282-301
		17	3	880	245- 633	5	290±2	275-303
		20	2	710	546-1247	6	289±3	270-306
		25	3	947	161- 352	7	286±3	274-301
	H. laevigata	12	1	494	137- 495	7	291±3	271-311
		17	1	553	77- 357	8	-	-
		17	2	698	659-1594	6	287±6	268-317
		17	3	937	386- 899	5	-	-
		20	2	757	597-1159	6	-	-
		25	3	1008	470-1006	7	292±2	282-307
	Hybrid	12	1	480	130- 305	6	295±4	281-320
		17	1	537	150- 459	6	-	-
		17	2	678	288- 882	5	295±3	275-305
		17	3	910	207- 804	6	-	-
		20	2	734	199- 654	6	-	-
		25	3	979	313- 625	6	291±3	273-307
Mar-16	Hybrid	12	3	965	190- 603	8	286±4	254-303
		17	1	544	193- 484	9	-	-
		20	2	698	224- 790	8	-	-
		25	1	480	65- 514	6	288±3	263-300
		28	3	929	56- 321	6	-	-
		30	2	743	118- 394	6	293±2	280-308
		32	2	788	122- 318	8	-	-

Table 4.2: Developmental day, corresponding effective accumulative temperature (EAT) [°C-h], and number of individuals (n) [ind ml^{-1}] of veliger larvae of *H. rubra*, *H. laevigata* and hybrid abalone at the start of thermal preference measurements.

	Nov-15			Dec-15			Mar-16		
Species	**Veliger day**	**EAT**	***n***	**Veliger day**	**EAT**	***n***	**Veliger day**	**EAT**	***n***
H. rubra	1	446	5	1	439	11	-	-	-
	2	628	6	1	493	11	-	-	-
	2	676	4	2	624	11	-	-	-
	3	842	13	2	681	29	-	-	-
	3	897	8	3	885	13	-	-	-
H. laevigata	-	-	-	1	394	25	1	483	4
	-	-	-	1	508	22	2	633	3
	-	-	-	2	687	20	2	670	3
	-	-	-	2	747	28	2	704	8
	-	-	-	3	921	22	3	880	7
	-	-	-	3	987	13	3	917	5
	-	-	-	-	-	-	3	954	5
	-	-	-	-	-	-	3	974	5
Hybrid	1	431	2	1	472	21	1	449	17
	2	663	7	2	687	16	1	466	8
	2	722	6	3	873	20	1	482	6
	3	901	5	3	936	16	1	503	6
	3	951	11	-	-	-	2	705	8
	-	-	-	-	-	-	2	738	7
	-	-	-	-	-	-	2	771	10
	-	-	-	-	-	-	3	909	17
	-	-	-	-	-	-	3	940	9
	-	-	-	-	-	-	3	976	10

Experimental design

Thermal preference and swimming speed

Larval acute thermal preference (T_{pref}) (Reynolds and Casterlin, 1979) and thermal influence on swimming speed (*U*) were measured in larvae from each of the three spawning events using a horizontal thermal gradient which enabled the larvae to choose a temperature while removing the influence of gravity and the tendency of abalone larvae to crowd at the tank surface (Prince *et al*., 1987). The thermal gradient was custom-built at the University of Tasmania and was similar to that described by Wiggins and Frappell (2000). The gradient consisted of a glass tube (54 cm length, 1 cm diameter, volume 42.42 ml) that was embedded in an aluminum block and further insulated with foam to minimise heat exchange with the environment. Both ends of the aluminum block were connected to Peltier devices so that temperatures could be regulated using a custom built electronic controller. Once the Peltier devices were turned on a thermal gradient was established in less than 30 min, similar to the thermal gradient used in Wiggins and Frappell (2000). The temperature gradient ranging from 12 to 25 °C was skewed (temperature [°C] = 25.074 - 0.040 - 0.004 × position in gradient, $r^2 = 0.998$ Fig. 4.1). Larval position within the gradient throughout the experimental period was observed through a thin viewing window in the aluminum block across the whole length of the glass tube. Three cameras (GoPro Hero 3, USA) were installed above the viewing window and enabled the whole gradient to be captured. The cameras took photos at a rate of one per min throughout all experiments.

Larvae of a single abalone group (*H. rubra, H. laevigata,* or their interspecies hybrid) were evenly dispersed throughout the glass tube using a syringe connected to 50 cm of aquarium tubing (see Table 4.2 for numbers of individuals ml^{-1}). Animals were left undisturbed for 1.5 h to distribute throughout the thermal gradient, after which 30-s videos were taken at 4 or 5 locations along the gradient (Celestron Deluxe Handheld Digital Microscope) to document the swimming speed of the animals. The temperature within the glass tube was subsequently measured every 2 cm using a thermocouple (a total of 25 readings) to quantify the thermal gradient after each experiment. In addition to the experimental trials, two control trials were conducted per day in which the temperature gradient was not established (i.e. Peltier devices turned off; uniform temperature of 19 °C, Fig. 4.1) to test whether the edges of the thermal gradient had any influence on larval distribution.

T_{pref} was determined for each trial by counting the number of larvae in each of the twenty five 2 cm sections in the photos captured 1.5 h after the larvae were introduced into the thermal

gradient. Numbers of larvae in consecutive sections were summed to determine the temperature range where 50% of the population occurred (Fig. 4.1). The average temperature of this range is considered herein to represent T_{pref}. Swimming speed [mm s^{-1}] was determined by manually tracking the distance travelled by larvae (n=6 to 10 for each location) between every frame (0.05 s) in the 30 s videos using the plugin MTrackJ for Image J (FIJI) (Abramoff *et al*., 2004; Myrick, 2009; Schindelin *et al*., 2015).

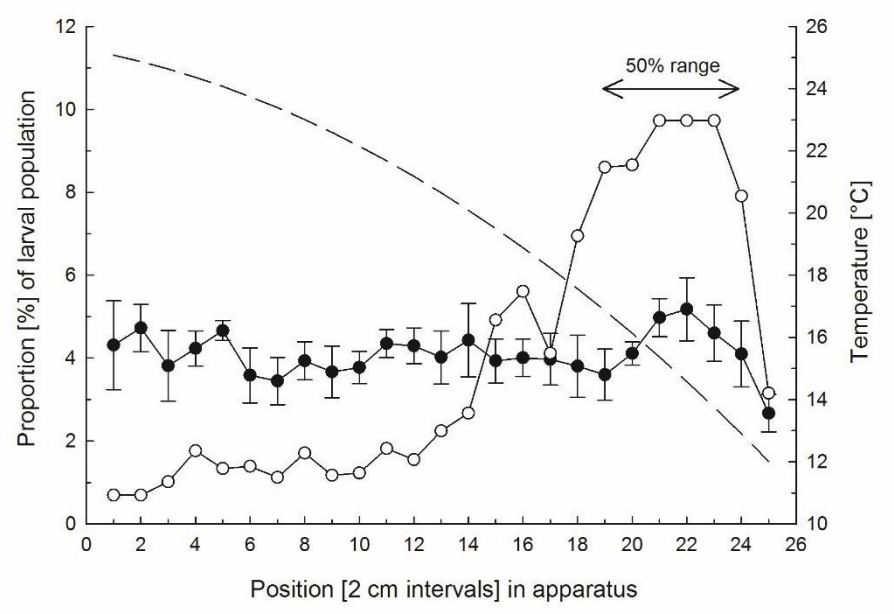

Fig. 4.1: Example of the distribution (% of population per 2 cm) of one abalone larval population (open circles, example of *H. rubra*, 493 °C-h) in the thermal gradient apparatus in the presence of a thermal gradient (dashed line). Black diamonds represent the average distribution during control runs (mean ± SE, n = 5) in the absence of a thermal gradient (uniform temperature of 19 °C, Peltier devices turned off). The horizontal arrow indicates 50% of larval population for which the average temperature was used as T_{pref} value.

Oxygen consumption rate

Oxygen consumption rate was measured on three days in December 2015 with veliger larvae of *H. rubra, H. laevigata,* and their interspecies hybrid, and on three days in March 2016 with hybrids only. Methods for measuring $\dot{M}O_2$ are described in Chapter 3. In brief, respiration chambers consisted of 24 wells (each 2.7 ml) within a galvanised aluminum plate. The aluminum plate was placed on a sensor dish reader (PreSens, Germany) which measured the oxygen content within each chamber at a rate of four readings per min. The measuring unit (aluminum plate and sensor dish reader) was placed in a temperature controlled room to manipulate temperature between temperature treatments and to ensure stable temperature during each experimental run. Individual respiration chambers were sealed with acrylic lids that contained a capillary hole and tube to prevent pressurizing the chamber during closure. Preliminary experiments showed that oxygen exchange through the capillary system was negligible. Veliger larvae swim constantly and stir the water such that a mechanical stirring mechanism was unnecessary. For each trial, batches of larvae from a single species group were transferred to respiration chambers filled with fresh seawater enriched with oxygen to a maximum of 120% O_2sat to ensure normoxic conditions once measurements commenced. The measurements commenced immediately after the chambers were hermetically sealed. The number of individuals per chamber varied between 56 and 1594 ind ml^{-1} (Table 4.1). A previous study has shown that different densities throughout this range do not influence larval abalone $\dot{M}O_2$ when calculated per individual (Chapter 3). Percentage air saturation was measured until oxygen dropped below 70% O_2sat (typically ~ 2 h at 32 °C and ~ 5 h at 12 °C). Sensors were calibrated in air-saturated seawater for 100% O_2sat and in sodium sulphite-saturated seawater for 0% O_2sat. Four chambers without individuals served as blanks to account for microbial respiration at each temperature. Blanks and $\dot{M}O_2$ (in pmol h^{-1} ind^{-1}) were calculated from the linear decrease in oxygen measured in each respiration chamber according to Eq. 1:

$$\dot{M}O_2 = \frac{\Delta FO_2}{\Delta t} \times (P_B - P_S) \times \beta_{O_2} \times Vol \times 0.2093 \qquad (1),$$

where ΔFO_2 is the difference in oxygen concentration, Δt is the difference in time (s), P_B is the barometric pressure (kPa), P_S is the saturation vapor pressure of water (kPa), β_{O_2} is the capacitance of water for oxygen, *Vol* is the chamber volume minus the larvae volume (L, assuming 1 g wet mass equals 1 ml and 1 larvae equals 1.4 µg), and 0.2093 is the fractional concentration of oxygen in water.

Abalone larvae are only spawned commercially twice per year in Australia, and the larvae develop very quickly with the veliger life stage only lasting 3 days at the commercial rearing temperature of 16 to 18 °C. As a result, $\dot{M}O_2$ experiments were conducted during December and March to gain a sufficient sample size. To determine the effect of temperature on $\dot{M}O_2$ of *H. rubra*, *H. laevigata* and hybrid larvae, experiments were conducted at 12, 17, 20, and 25 °C in December. Further, to determine the temperature where $\dot{M}O_2$ becomes compromised in the most important commercial abalone, i.e. hybrid larvae, $\dot{M}O_2$ of hybrids was determined at the aforementioned temperatures plus the additional temperatures of 28, 30, and 32 °C in March 2016. The $\dot{M}O_2$ data were collected over 3 days because it was logistically impractical to measure $\dot{M}O_2$ at all temperatures within one day. It was not expected that larval $\dot{M}O_2$ would vary across this time because hybrid larvae maintain constant $\dot{M}O_2$ during their 3 days as veligers at a constant temperature of 16 °C (Chapter 3). Nevertheless, two experimental considerations further minimised the potential effect of experimental day on $\dot{M}O_2$. First, during the December experiment, the $\dot{M}O_2$ of larval *H. rubra*, *H. laevigata* and hybrids was measured at 17 °C on each of the three consecutive days to determine if $\dot{M}O_2$ at a stable temperature changed throughout the three experimental days. Second, the order at which $\dot{M}O_2$ was determined at temperatures other than 17 °C was randomised between December and March experiments. In December, larval $\dot{M}O_2$ was measured at 12, 20, and 25 °C at the first, second and third day of veliger larvae development, respectively (n= 5 to 8 replicates for each abalone group and temperature; Table 4.1). In March, $\dot{M}O_2$ of hybrids was measured at 25, 20, and 12 °C at the first, second and third day, respectively (n= 5 to 9 replicates for each temperature; Table 4.1). The additional temperature treatments to determine the temperature where hybrid $\dot{M}O_2$ becomes compromised were conducted in March 2016 as follows: 17 °C on the first day, 30 and 32 °C on the second day and 28 °C on the third day (Table 4.1). After each trial, larvae were preserved in 10% formalin and transported to CSIRO, Hobart, Australia, where they were counted and veliger SL was measured (Table 4.1).

Statistical analyses

Statistical analyses were conducted using SigmaPlot 13.0. Shapiro-Wilk tests were used to test for normality and Brown-Forsythe tests were used to test for equal variances. Multiple pairwise comparisons were conducted with Student-Newman-Keuls tests. Significance was considered at $p < 0.05$.

Differences between the distributions of the larvae with and without a thermal gradient were tested using Chi-square tests. Then, differences in T_{pref} between species and developmental days were examined using two-way ANOVA.

Measures of *U* data were pooled at each developmental day for each group of abalone. Then, the influence of temperature, developmental days and group of abalone on *U* was tested using a three-way ANOVA.

The $\dot{M}O_2$ data of hybrids from the March population were log-transformed to satisfy requirements for normality and a one-way ANOVA was conducted to determine the temperature at which $\dot{M}O_2$ became compromised and started to decline. For $\dot{M}O_2$ data at 17 °C for each group of abalone from the December population, a one-way ANOVA was conducted to test if $\dot{M}O_2$ changed during development. Oxygen consumption rate did not change during development at 17 °C and data were pooled afterwards for each group of abalone. $\dot{M}O_2$ data at 12 to 25 °C for the December and March populations were used to test for differences between temperatures and groups of abalone. For this data normality was not satisfied and a non-parametric Scheirer-Ray Hare test was conducted (Scheirer *et al*., 1976; Sokal and Rohlf, 1995).

Results

Thermal preference

Veliger distributions were significantly different with and without the thermal gradient (Chi-square test, $p < 001$; Fig. 4.1). The T_{pref} was similar between groups of abalone and increased significantly during development from one day to three day old veligers (two-way ANOVA, group of abalone $F_{2,34} = 1.26$, $p > 0.05$; development $F_{2,34} = 21.14$, $p < 0.001$; Fig. 4.2). Values of T_{pref} increased from 15.9 ± 1.0 °C to 19.5 ± 0.1 °C in *H. rubra* ($p < 0.01$), from 17.0 ± 0.3 °C to 20.0 ± 0.6 °C in *H. laevigata* ($p < 0.02$) and from 16.0 ± 0.2 °C to 20.2 ± 0.8 °C in hybrids ($p < 0.01$; Fig. 4.2).

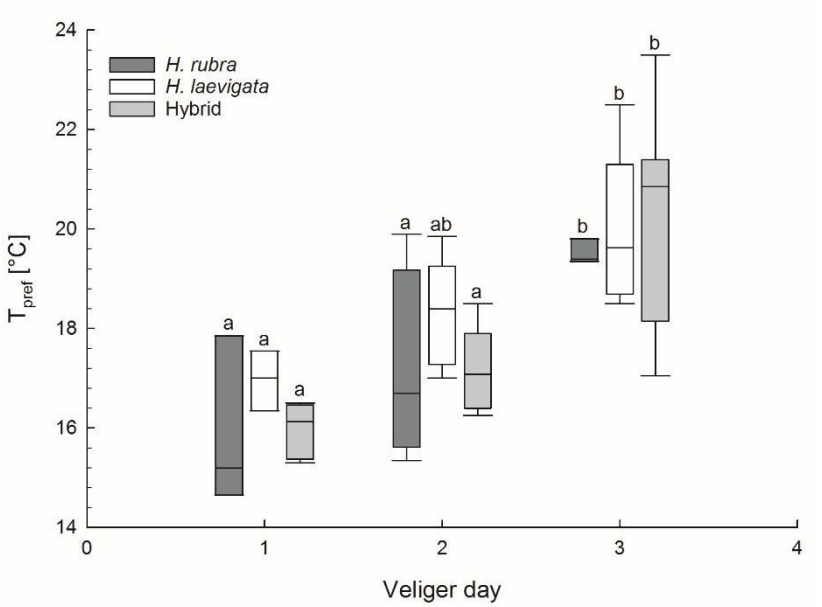

Fig. 4.2: Thermal preference (T_{pref}, °C) of *H. rubra* (dark grey bars), *H. laevigata* (open bars), and hybrid larvae (light grey bars) during veliger development [days]. The box shows the 95% range and is offset for visual clarity. The horizontal line indicates the median. Whiskers represent the lowest and highest values. Sample sizes are in Table 4.1. Different *lowercase letters* indicate significant differences between veliger days within each abalone group (two-way ANOVA, Student-Newman-Keuls (SNK), $p < 0.05$). There was no significant difference between either of the two pure species or the hybrid at each veliger day (two-way ANOVA, SNK, $p > 0.05$).

Swimming speed

The swimming speed *U* [mm s^{-1}] of veligers was significantly influenced by the abalone group (*H. rubra, H. laevigata,* or hybrid), temperature and developmental day (three-way ANOVA, group of abalone $F_{2,593} = 14.96$, $p < 0.001$; temperature $F_{3,593} = 261.25$, $p < 0.001$; day $F_{2,593} = 13.00$, $p < 0.001$; Fig. 4.3). There were significant interactions between temperature and group of abalone, between temperature and developmental day and between group of abalone, temperature and developmental day (three-way ANOVA, temperature/group of abalone $F_{6,593} = 7.01$, $p < 0.001$; temperature/day $F_{6,593} = 3.69$, $p = 0.001$; group of abalone/temperature/day $F_{12,593} = 2.37$, $p = 0.006$). For *H. laevigata* veliger larvae, *U* at 12 °C (1.96 ± 0.14 mm s^{-1}), 17 °C (2.18 ± 0.12 mm s^{-1}), 20 °C (2.75 ± 0.13 mm s^{-1}), and 25 °C (3.97 ± 0.14 mm s^{-1}) was similar across developmental days ($p > 0.05$), with values significantly higher at 25 °C ($p < 0.001$; Fig. 4.3b). For *H. rubra*, *U* at 12, 17, and 20 °C was similar between developmental days ($p > 0.05$) and averaged 1.99 ± 0.14, 2.38 ± 0.10, and 2.91 ± 0.10 mm s^{-1}, respectively. At 25 °C, *U* of *H. rubra* was increased during veliger development and was lowest in one day old veligers (4.10 ± 0.17 mm s^{-1}), significantly higher in two day old veligers (5.06 ± 0.17 mm s^{-1}, $p < 0.005$) and highest in three day old veligers (5.93 ± 0.19 mm s^{-1}, $p < 0.001$). Similar to *H. laevigata*, *U* of *H. rubra* was not significantly different between 12 and 17 °C ($p > 0.05$) but was significantly elevated at 25 °C ($p < 0.001$) at all tested veliger days (Fig. 4.3a). For hybrid larvae, *U* at 12 to 20 °C was similar between developmental days ($p > 0.05$) and increased significantly between each temperature ($p < 0.03$) from 1.66 ± 0.10 mm s^{-1} at 12°C to 2.38 ± 0.14 mm s^{-1} at 17 °C and 3.38 ± 0.14 mm s^{-1} at 20 °C. At 25 °C, *U* of hybrids increased across development like those of *H. rubra* and was significantly lower in one day old veligers (4.19 ± 0.15 mm s^{-1}) in comparison to three day old veligers (6.09 ± 0.21 mm s^{-1}) ($p < 0.001$; Fig. 4.3c).

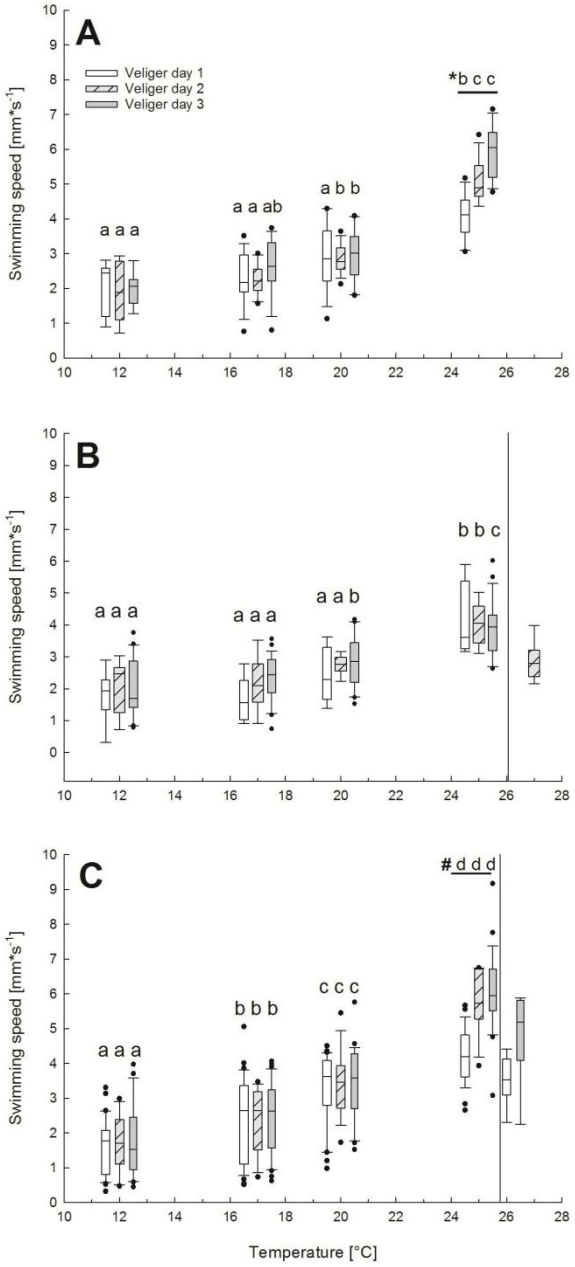

Fig. 4.3: Swimming speed (*U*, mm s^{-1}) of *H. rubra* (A), *H. laevigata* (B), and hybrid larvae (C) at veliger day 1 (white), day 2 (striped), and day 3 (grey) measured at 12, 17, 20, and 25 °C. The meaning of boxes and whiskers are as described in Fig. 4.2. Dots symbolise outliers. n = 7 to 37. Different *lowercase letters* indicate significant differences between temperature treatments for a given veliger day. # = significant differences between veliger day 1 and 3 at a given temperature. * = significant differences between all veliger days at a given temperature. *U* above 25 °C, shown to the right of the vertical line, were not collected for all abalone types and were therefore not included in the statistical analysis (three-way ANOVA, Student-Newman-Keuls, $p < 0.05$).

Oxygen consumption rate

The $\dot{M}O_2$ [pmol O_2 h^{-1} ind^{-1}] of *H. rubra*, *H. laevigata*, and hybrids measured in December at 17 °C was stable over the three day developmental period (one-way ANOVA, *H. rubra* $F_{2,14} = 0.23$, $p > 0.05$; *H. laevigata* $F_{2,16} = 1.06$, $p > 0.05$; hybrids $F_{2,14} = 0.56$, $p > 0.05$; Fig. 4.4). For the December populations, $\dot{M}O_2$ was influenced by temperature and group of abalone (two-way ANOVA, temperature $H_{3,126} = 86.91$, $p < 0.001$; group of abalone $H_{3,126} = 16.09$, $p < 0.001$; Fig. 4.5). The interaction between the two factors was not significant (two-way ANOVA, $H_{9,126} =$, $p > 0.05$; Fig. 4.5). For both *H. rubra* and hybrids the $\dot{M}O_2$ from the December population increased gradually with increasing temperature from 12 to 25 °C ($p > 0.05$), while $\dot{M}O_2$ of *H. laevigata* was similar between 17 and 20 °C ($p > 0.05$) and between 20 and 25 °C ($p > 0.05$; Fig. 4.5). At 12, 17, and 20 °C all abalone had a similar $\dot{M}O_2$ with average values of 73.65 ± 1.50, 93.08 ± 1.81, and 114.85 ± 3.83 pmol O_2 h^{-1} ind^{-1}, respectively ($p > 0.05$). At 25 °C, $\dot{M}O_2$ of *H. laevigata* (118.31 ± 4.55 pmol O_2 h^{-1} ind^{-1}) was significantly lower than that of *H. rubra* (151.21 ± 9.61 pmol O_2 h^{-1} ind^{-1}) and hybrids (147.53 ± 7.71 pmol O_2 h^{-1} ind^{-1}, $p < 0.05$; Fig. 4.5).

For the March spawning, hybrid $\dot{M}O_2$ increased gradually from 12 °C (74.42 ± 2.20 pmol O_2 h^{-1} ind^{-1}) to a maximum value at 25 °C (176.73 ± 6.19 pmol O_2 $h^{-1}ind^{-1}$, $p < 0.001$), after which it decreased until 28 °C (147.79 ± 9.78 pmol O_2 h^{-1} ind^{-1}, $p = 0.017$). $\dot{M}O_2$ remained stable from 28 to 32 °C (average value = 133.86 ± 4.43 pmol O_2 h^{-1} ind^{-1}, $p > 0.05$) (one-way ANOVA, temperature $F_{6,44} = 31.19$, $p < 0.001$; Fig. 4.6). In comparison to larvae of *H. rubra*, *H. laevigata* and hybrids from the December population, larvae of hybrids from the March population had a similar $\dot{M}O_2$ at 12 °C ($p > 0.05$) but 20% increased values at 17 and 20 °C. At 25 °C hybrid larvae from March had 15% higher $\dot{M}O_2$ values in comparison to *H. rubra* and hybrid larvae from December and 30% higher values in comparison to *H. laevigata* larvae from December ($p < 0.05$) (two-way ANOVA, temperature $H_{3,126} = 86.91$, $p < 0.001$; group of abalone $H_{3,126} = 16.09$, $p < 0.001$; Fig. 4.5 and 4.6).

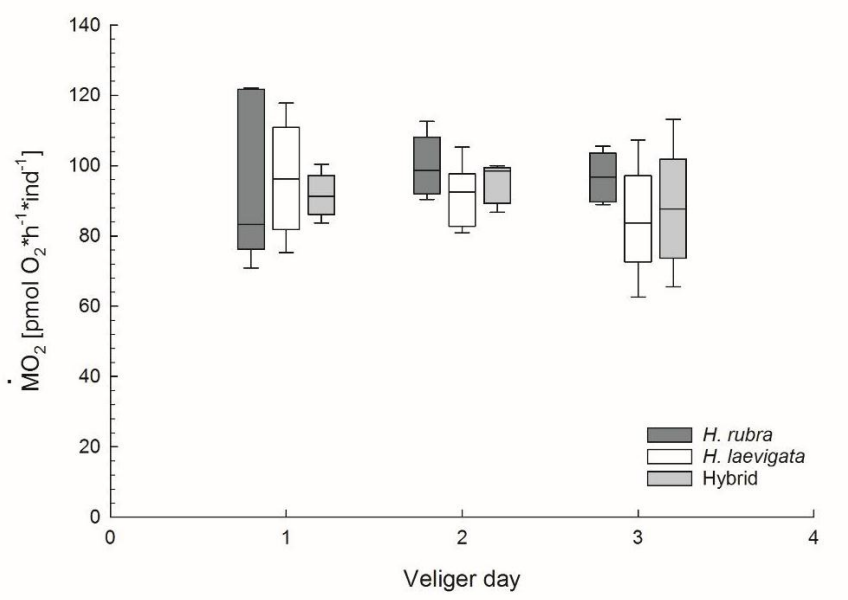

Fig. 4.4: Oxygen consumption rate ($\dot{M}O_2$, pmol O_2 h^{-1} ind^{-1}) of *H. rubra* (dark grey bars), *H. laevigata* (open bars), and their interspecies hybrid (light grey bars) during veliger larvae development [days] at 17 °C. The meaning of boxes and whiskers are as described in Fig. 4.2. n = 5 to 8.

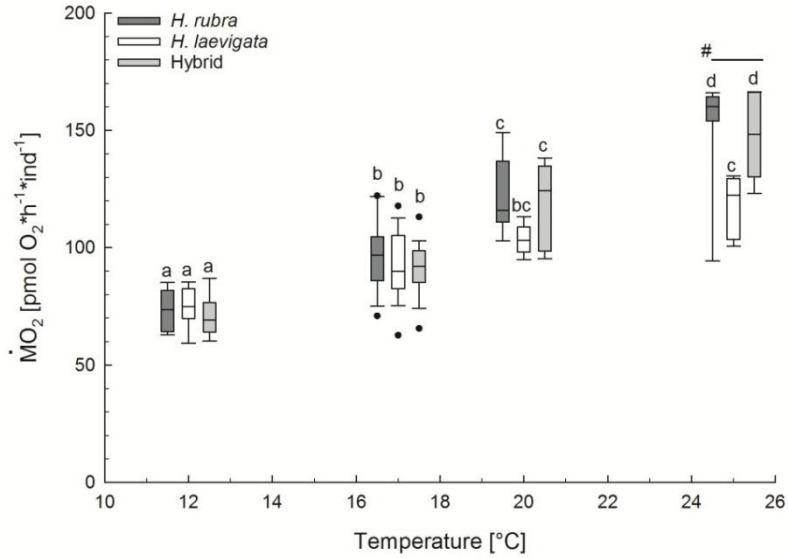

Fig. 4.5: Oxygen consumption rate ($\dot{M}O_2$, pmol O_2 h^{-1} ind^{-1}) of *H. rubra* (dark grey bars), *H. laevigata* (open bars), and hybrids (light grey bars) measured at 12, 17, 20, and 25 °C. See Table 4.1 for veliger developmental day and sample size. The meaning of boxes and whiskers are as described in Fig. 4.2. Dots symbolise outliers. Different *lowercase letters* indicate significant differences between temperature treatments for a given group of abalone. # indicates significant differences between species at a given temperature (non-parametric Scheirer-Ray Hare, $p < 0.05$). There was no significant interaction between temperature and type of abalone (non-parametric Scheirer-Ray Hare, $p > 0.05$).

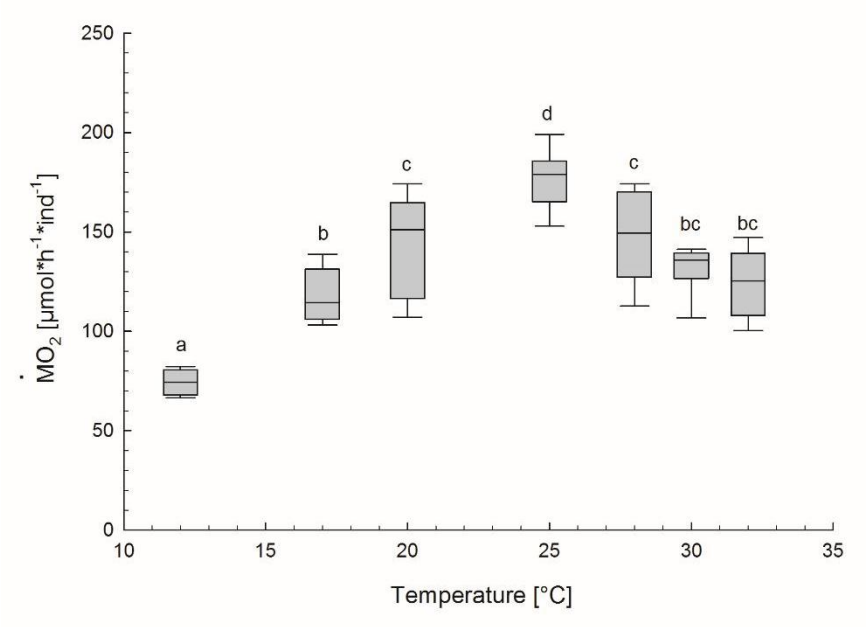

Fig. 4.6: Oxygen consumption rate ($\dot{M}O_2$, pmol O_2 h^{-1} ind^{-1}) of hybrid larvae from the March population at various temperatures [°C]. See Table 4.1 for veliger developmental days and sample size. The meaning of boxes and whiskers are as described in Fig. 4.2. Different *lowercase letters* indicate significant differences between temperatures.

Discussion

Thermal preference (T_{pref}) of *H. rubra, H. laevigata*, and hybrid larvae ranged from 16 °C in early veligers to 20 °C in late veligers (Fig. 4.2). Ecological conclusions are difficult to draw because few larvae have been found in the wild (Breen and Adkins, 1980; McShane *et al*., 1988; Babcock and Keesing, 1999). Larvae in this study are from third generation selected broodstock, with founder populations originating mainly from Victorian coastal and bay populations of abalone. In this region, minimum and maximum sea temperatures are 11.8 and 25.4 °C, respectively, with an annual mean of 18.6 °C (Australian Government Bureau of Meteorology, 2016). The T_{pref} of larvae determined in this study and T_{pref} measured in adult *H. rubra* and *H. laevigata* of 17 and 19 °C, respectively, fit within the natural temperature range of the founders (Gilroy and Edwards, 1998).

All three abalone groups in this study increased T_{pref} during larval development (Fig. 4.2). Abalone veligers close to settlement might select warmer waters to decrease the time needed to metamorphose, subsequently increasing their chance of survival and successful settlement. Developmental time is strongly dependent on temperature and individuals will pass the

vulnerable metamorphosis stage more rapidly by selecting higher temperatures at the end of the larval phase (Leighton, 1972). Larval amphibians also increase T_{pref} during development and select warmer waters at the settlement stage to facilitate faster metamorphosis and reduce predation (Floyd, 1984; Dupré and Petranka, 1985; Wollmuth *et al*., 1987). Similar to amphibians, metamorphosing abalone larvae are likely to be more susceptible to predation because they have lower mobility in comparison to swimming larvae and are less protected than juveniles and adults which have protective juvenile/adult shells (Crofts, 1937).

Ectotherms often select temperatures below their maximal physiological capacity (Martin and Huey, 2008). Although a maximum level was not determined for *H. rubra* and *H. laevigata*, the decline in hybrid $\dot{M}O_2$ at temperatures higher than 25 °C may indicate that this temperature is close to the upper physiological limit for hybrids (Fig. 4.6). In agreement, swimming speeds (*U*) of hybrid and *H. laevigata* larvae also decreased at temperatures higher than 25 °C (higher temperature values not determined for *H. rubra*) (Fig. 4.3). At 20 °C, *U* was similar not only between species but also across development, while at 25 °C *H. rubra* and hybrids displayed an increase in *U* with increasing development. The latter is not a simple consequence of increasing body size, because larvae do not feed exogenously and hence do not gain mass during larval development (Table 4.1). The increasing *U* across development at 25 °C may instead be related to the larvae acquiring an enhanced motivation to find suitable surfaces for settlement. Yet, finding settlement habitats at such high temperatures may not be favourable for survival. Indeed, larvae of *H. sorenseni* Bartsch inhabit waters with temperatures of 14 to 20 °C and while development was most rapid at 20 °C high mortality occurred during juvenile development at that temperature (Leighton, 1972; Leighton *et al*., 1981).

It remains unclear why the increase in *U* at 25 °C during the development of *H. rubra* and hybrids in the present study was not observed for *H. laevigata*. A potential reason could be associated with energy storages which become limiting at the end of larval development (Shilling *et al*., 1996; Moran and Manahan, 2003). It may be that *H. laevigata*, in comparison to *H. rubra* and hybrids (hybrids produced via *H. rubra* ova), has less energy reserves available at the end of larval development and hence is not able to increase *U* at such high temperatures. In accordance with this hypothesis, a two-fold difference in lipid reserves between coexisting larvae has been reported for *H. sorenseni* and the green abalone, *H. fulgens* (Moran and Manahan, 2003). A study addressing the energy reserves in *H. rubra*, *H. laevigata*, and hybrid abalone larvae could shed light on this idea.

The $\dot{M}O_2$ of veliger larvae decreased with declining temperatures to the lowest tested temperature of 12 °C (Fig. 4.5). This suggests that the lower physiological limit is below 12 °C which is in accordance with previous reports (Grubert and Ritar, 2004). The biological zero point (BZP) is the theoretical threshold below which larval development is stunted and is reported as 7.8 °C for *H. rubra* and 7.2 °C for *H. laevigata* (Grubert and Ritar, 2004). Grubert and Ritar (2004), however, determined the BZP of larvae originating from wild-caught broodstock from cooler Tasmanian waters, whereas larvae in the present study originated from cultured broodstock and were raised in warmer conditions, which may influence the BZP. In addition, *U* of *H. rubra* veligers as well as *U* of *H. laevigata* veligers was similar between 12 and 17 °C (Fig. 4.3) and larval swimming behaviour was extremely sluggish and circular at 12 °C in comparison to the more directional swimming behaviour at higher temperatures. This suggests that 12 °C is approaching the lower temperature limit for swimming ability in larvae. Similar observations have been reported for white abalone, *H. sorenseni* larvae which developed but did not settle successfully during temperature exposures of 10 to 12 °C (Leighton, 1972). Further, postlarvae of the red abalone, *H. rufescens*, the pink abalone, *H. corrugata*, and the green abalone, *H. fulgens* were also able to survive at temperatures of 10 to 12 °C but were not able to right themselves when turned over (Leighton, 1974). White, red, pink and green abalone inhabit waters with temperatures of 14 to 20 °C, which are similar to the temperatures in the Victorian waters where the founder broodstock for larvae from the present study were sourced. It may be possible that the temperatures that resulted in impaired swimming ability in this study and settling success in white abalone as well as righting ability in red, pink and green abalone are indicative of the lower thermal limit for physiological function and thus constrain distribution within these temperate abalone species.

At 17 to 25 °C, hybrids from the March population had higher $\dot{M}O_2$ in comparison to hybrids (and pure species) from the December population (Fig. 4.4 and 4.6). For example, hybrid MO_2 at 17 °C was 91 pmol h^{-1} ind^{-1} for the December population and 118 pmol h^{-1} ind^{-1} for the March population. Differences in $\dot{M}O_2$ between larval populations of *H. rubra*/*H. laevigata* hybrids from the same farm have been reported previously (Chapter 3). $\dot{M}O_2$ varied between 99 and 146 pmol h^{-1} ind^{-1} at 16 °C and it was suggested that the differences may be due to heritable and/or maternal variation (Chapter 3). Indeed, one third of the variation in veliger *U* and growth rates was caused by genetic and maternal factors in the common slipper snail, *Crepidula fornicata* Linnaeus (Hilbish *et al*., 1999). High levels of genetic variation for

veliger growth rates have also been reported for other mollusks, such as the oyster, *Crassostrea virginica* Gmelin, the hard clam, *Mercenaria mercenaria* (Linnaeus), and the Manila clam, *Ruditapes philippinarum* Adams and Reeve (Newkirk *et al*., 1977; Hilbish *et al*., 1993; Yan *et al*., 2014).

In summary, results of the present study do not support the original hypothesis that T_{pref} is different between groups of abalone. Findings of the present study suggest that the current aquaculture industry best practices for rearing pure and hybrid groups of early-stage larvae at 16 to 18 °C are appropriate (Heasman and Savva, 2007; L. McPherson, Jade Tiger Abalone, pers. com., November 2014). The T_{pref} of one and two day old veligers was between 15.9 and 18.3 °C (Fig. 4.2). These temperatures represent the approximate average temperature range of physiologically limiting low and high temperatures of 12 and 25 °C, respectively. Further, *U* of veligers at T_{pref} (approx. 17 °C) was constant during development (Fig. 4.3). Three day old veliger larvae are close to settlement and preferred higher temperatures between 19.5 and 20.2 °C, in comparison to one and two day old veligers. This may indicate that temperatures in the hatchery could be slightly increased during later stage larval development prior to settlement. Yet, this suggestion should be considered with care until similar studies are completed with settled larvae and early juveniles. Adult T_{pref} has been reported as 17 and 19 °C in *H. rubra* and *H. laevigata*, respectively (Gilroy and Edwards, 1998). This may indicate that T_{pref} may decrease after metamorphosis in abalone as demonstrated in other aquatic organisms (Dupré and Petranka, 1985; Wollmuth *et al*., 1987).

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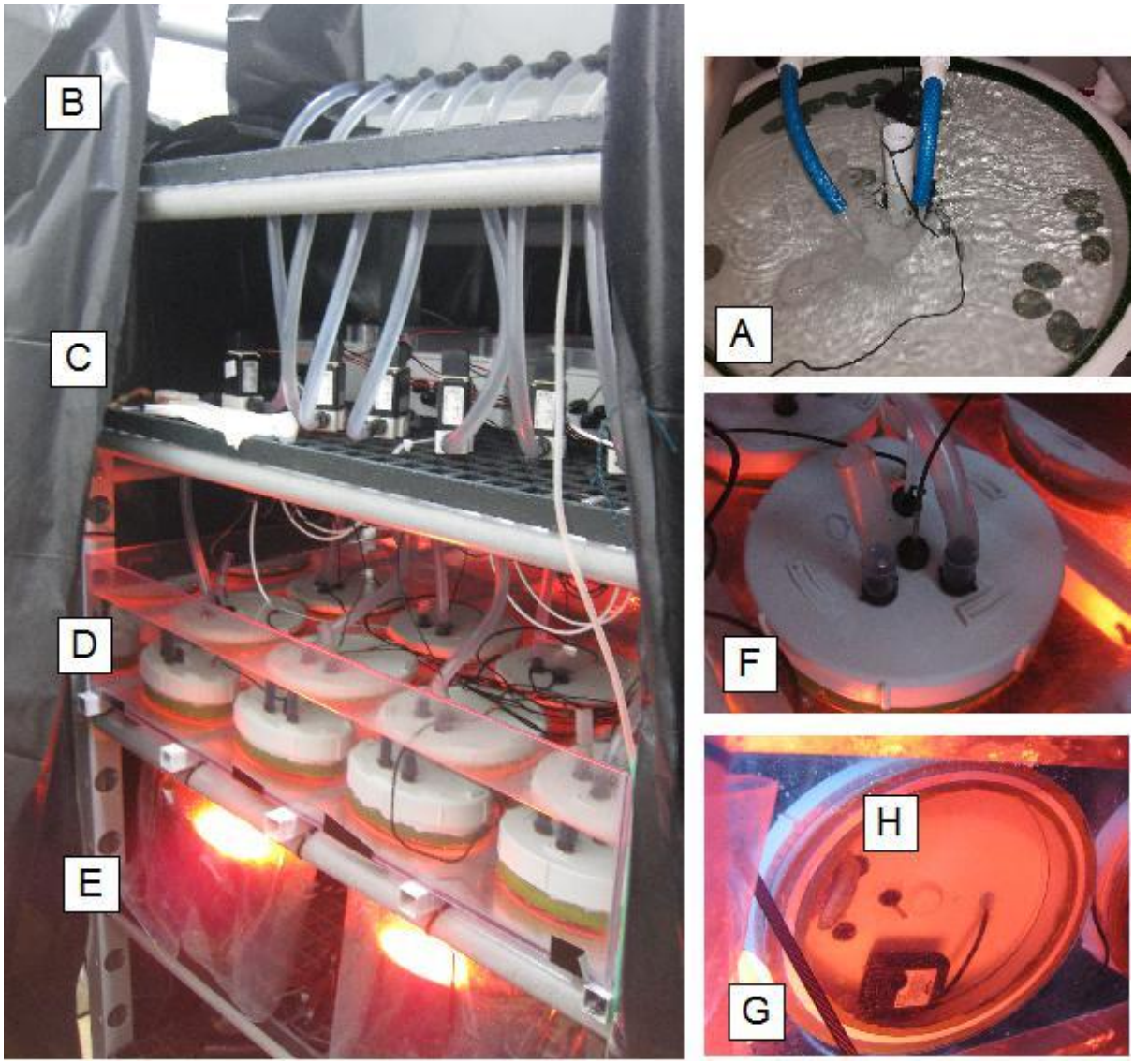
CHAPTER 5: Movement energetics of juvenile abalone

In preparation:

Alter K, Andrewartha SJ, Morash AJ, Clark TD, Elliott NG (*in prep*) Aerobic and anaerobic movement energetics of hybrid and pure parental species.



Experimental set-up (B-H) used in this chapter following acclimation of abalone to abiotic conditions (A). The set-up consisted of level 1 (B): water reservoir, level 2 (C): solenoid valves, level 3 (D): respiration chambers in a water bath and level 4 (E): red light source and cameras. Respiration chambers were PVC pipes with screw caps with water in- and outflow tubes as well as oxygen and temperature sensors (F). The bottom of the chamber was made from glass (G) to be able to observe abalone movement (H).

Abstract

The hybrid between blacklip abalone, *H. rubra*, and greenlip abalone, *H. laevigata* shows enhanced growth in comparison to both pure parental species, yet the underlying mechanisms remain unknown. Abalone frequently rely on anaerobic energy even in the presence of oxygen and especially during activity. Here, it was tested whether the growth advantage of the hybrid compared with pure parental species may be linked with differences in movement and/or use of aerobic versus anaerobic energy. Hybrids, *H. rubra*, and *H. laevigata*, of 12 to 15 months of age were acclimated for 2 to 3 weeks to a combination of control (16 °C, 100% O_2sat) and typical summer conditions (23 °C, and/or 70% O_2sat) using a factorial crossed design. Movement and oxygen consumption rates ($\dot{M}O_2$) were then measured over a 48 h period at each acclimation condition (chronic exposure). Critical oxygen tension (P_{crit}) was then determined during an acute decrease in oxygen saturation. Tauropine dehydrogenase (TDH) and lactate dehydrogenase (LDH) activities were determined using muscle tissue from acclimated hybrids and pure species to understand anaerobic capacity. No circadian patterns were observed in either movement or $\dot{M}O_2$. Movement during chronic exposure was similar between acclimation conditions within each of the three abalone groups, although *H. rubra* generally exhibited greater movement than the other two groups. A marked movement increase below P_{crit} (~ 40% O_2sat) was observed for hybrids and *H. laevigata* but not *H. rubra*. During the chronic exposure, $\dot{M}O_2$ of all abalone groups was similar between 100% and 70% O_2sat. Yet, when oxygen levels for the 70% O_2sat-acclimated abalone were raised to 100% O_2sat before P_{crit} determinations commenced, $\dot{M}O_2$ increased above that of conspecifics acclimated to 100% O_2sat. Chronic exposure to 70% O_2sat resulted in increased TDH activities at both temperatures and increased LDH activities at 23 °C in hybrids and *H. laevigata* when compared to 100% O_2sat. All three abalone groups showed evidence of supplementing their aerobic metabolism using anaerobic pathways, even at oxygen levels above their P_{crit} and to an enhanced extent at the higher temperature. The observed differences in movement, anaerobic enzymes and $\dot{M}O_2$ between hybrids and pure species, were indicative but not marked enough to support the original hypothesis that hybrids have an energetic advantage over pure species.

Key words: Locomotion, anaerobic enzyme activity, abalone, critical oxygen level

Introduction

Shellfish with desirable characteristics such as improved growth, superior behavioural traits, and higher resistance to environmental stress are produced in aquaculture through selective breeding and hybridization programmes (Leighton and Lewis, 1982; Elliott, 2000; Cheng *et al*., 2006; Kube *et al*., 2007; Hamilton *et al*., 2009). In Australian abalone, an interspecies hybrid that grows 25% faster and survives better during live transport than parental pure species is produced from blacklip abalone, *H. rubra,* and greenlip abalone, *H. laevigata* (A. Krsinich, JTA, pers. comm., November 2013; P. Kube, CSIRO, pers. comm., 2014). The behaviour of the hybrid is also better suited to aquaculture, unlike its parental species *H. rubra*, because it does not escape grow-out tanks (Guo, 2009). The advantages of the hybrid, however, have not been scientifically demonstrated and little is known about the physiological mechanisms underlying the better performance of hybrids.

Abalone are generally sedentary and slow-moving, yet they show an array of behaviours including crawling, righting after being flipped, shell twisting and clamping when threatened, and foot lifting for trapping drift algae for food (Donovan, 1998). Behaviour is among the first characteristics that change in response to captivity and domestication (Huntingford, 2004; Lachambre *et al*., 2017). On land-based aquaculture farms, predators are absent and artificial food pellets are fed in high quantities reducing the need to hide and forage (Cenni *et al*., 2009). Nonetheless, cultured abalone move within grow-out tanks generally against the water flow and with less movement when held under constant darkness (A. Krsinich, JTA, pers. comm., 2014). Further, aquaculture farmers report that *H. rubra* is more active than *H. laevigata*, which is similar to observations from the wild, and that the hybrid possesses intermediate movement behaviour (Shepherd, 1973; A. Krsinich, JTA, pers. comm., 2014). Determining movement patterns and overall activity may reveal an advantage of the hybrid over pure species, in that lower activity of hybrids may provide an energetic advantage and a higher scope for growth.

Activity in gastropods account for a large energy expenditure due to costs of crawling and mucous production (Calow, 1974; Denny, 1980; Edwards and Welsh, 1982; Horn, 1986; Peck *et al.*, 1987; Donovan *et al*., 1999). Yet, not all energy used for locomotion may be lost because for some species mucous enhances growth of the biofilm on which gastropods feed (Calow, 1974). Further, it has been demonstrated for some intertidal gastropods that individuals follow the mucous trails of conspecifics which results in a reduction in energy expenditure for locomotion because the follower individual reduces its moucus production

(Davies and Blackwell, 2007). Abalone rely frequently on anaerobic energy pathways, which have been estimated to fuel more than 50% of the total cost of transport in *H. kamschatkana* (Donovan *et al*., 1999). The major anaerobic enzymes produced by abalone are tauropine dehydrogenase (TDH) and lactate dehydrogenase (LDH), which catalyse the reaction of pyruvate to tauropine and D-lactate, respectively (Gäde, 1988; Baldwin *et al*., 1992). The relative abundance of these enzymes is tissue-specific with larger quantities of TDH in the adductor muscle and LDH in the foot muscle. As a result, TDH is the predominant enzyme used for anaerobic energy production during functional hypoxia that results from exercise, while LDH dominates during exposure to environmental hypoxia (Gäde, 1988; Baldwin *et al*., 1992). Producing energy via opine and lactate pathways is inefficient in comparison to aerobic pathways (Lee and Lee, 2011). Hence, lower anaerobic enzyme activity in hybrids, for example, could translate to an energetic advantage and faster growth.

Oxygen consumption rates ($\dot{M}O_2$) provide insight into aerobic energy production and may also reveal reasons for improved growth rates of the hybrid. Oxygen consumption rates measured during uncontrolled movement have been termed routine $\dot{M}O_2$, while resting $\dot{M}O_2$ describes $\dot{M}O_2$ at zero activity (Prosser, 1961). Controversy exists over whether higher or lower resting $\dot{M}O_2$ results in improved growth. The "compensation hypothesis" predicts that individuals with lower resting $\dot{M}O_2$ due to lower maintenance costs can channel more energy into growth (reviewed by Burton *et al*. (2011)). In contrast, the "increased intake hypothesis" suggests that individuals with higher resting $\dot{M}O_2$ can gain more energy per unit time and thus grow faster. It has been suggested that the latter hypothesis might be only valid for environments with an excess food supply (reviewed by Burton *et al*. (2011)), which is the case in aquaculture facilities. In agreement, cultured fish with a higher resting $\dot{M}O_2$ grow faster when sufficient food is available (Norin *et al*., 2016). It is unknown which, if any, hypothesis is valid for abalone and may help to explain hybrid vigour because hybrid and pure abalone species resting $\dot{M}O_2$ has not yet been compared.

Further, $\dot{M}O_2$ can give insight into hypoxia sensitivity (Pörtner *et al*., 2010). Abalone are oxygen regulators, i.e. they maintain their $\dot{M}O_2$ until a critical oxygen level (P_{crit}) (Jan and Chang, 1983). Below the P_{crit}, $\dot{M}O_2$ drops rapidly and individuals increasingly rely on anaerobic energy pathways (Pörtner and Grieshaber, 1993). A lower P_{crit} in hybrids, for example, may lead to higher energy efficiency due to aerobic energy production over a wider oxygen range. A relative insensitivity to dissolved oxygen levels may be particularly advantageous in unstable abiotic environments, such as aquaculture farms.

In Australian abalone aquaculture, water is sourced from the nearby ocean and as a result the abiotic factors remain largely uncontrolled (reviewed in Chapter 2). Temperature fluctuates according to environmental conditions. Abalone in this study were sourced from the largest abalone farm in Victoria, Australia, where temperature ranges from a minimum of 9 °C in winter and a maximum of 23 °C in summer. The dissolved oxygen level of the incoming water is usually fully saturated, yet oxygen commonly decreases to approximately 70% O_2sat along the abalone tank (Wassnig *et al.*, 2009; Wassnig *et al.*, 2010; Alter unpubl. data). This unstable environment may be stressful for abalone which may increase movement to avoid suboptimal conditions (Jan and Chang, 1983; Harris *et al.*, 1999). Hence, a lower abiotic sensitivity of hybrids in comparison to pure species may result in lower activity of the hybrid, reduced reliance on anaerobic pathways, and hence, a higher scope for growth.

To assess these possibilities, movement, $\dot{M}O_2$, and TDH and LDH activities were examined in this study in hybrid abalone, *H. rubra*, and *H. laevigata* following acclimation to all four combinations of control (16 °C, 100% O_2sat) and summer conditions (higher temperature of 23 °C, and lower dissolved oxygen of 70% O_2sat). Movement and $\dot{M}O_2$ were measured for two days at stable acclimation conditions and then subsequently during acute oxygen decrease. It was hypothesized that hybrids are less active than pure parental species and thus have lower TDH activities. Further, is was predicted that hybrid movement and $\dot{M}O_2$ are least affected by low oxygen conditions and thus hybrids have lower LDH activities. Moreover, hybrids were predicted to have a higher resting $\dot{M}O_2$ that would encourage growth in food-plentiful aquaculture environments. Together, these characteristics would result in a more optimal energetic strategy of the hybrid under aquaculture conditions and lead to the observed growth heterosis.

Methods

Experimental animals

Individually tagged (Hallprint tags) hybrids (n = 89), *H. rubra* (n = 90) and *H. laevigata* (n = 84) were sourced from Jade Tiger Abalone (JTA) in Indented Head, Victoria. Hybrid abalone were from *H. rubra* females and *H. laevigata* males. All three types of abalone were from the JTA selective breeding program and were sourced from one family to reduce the influence of genetic variation on behaviour and physiology. Abalone were transported from JTA to the CSIRO laboratories in Hobart, Tasmania according to standard industry shipping practices. In brief, abalone were levered off the substrate in the grow-out tanks with a blunt spatula and

transferred to purge tanks with a constant temperature of 16 °C and no food supply. After two days, the abalone were transferred into plastic bags filled with oxygen at 300% O_2sat and placed in a styrofoam box. The box was equipped with ice packs to maintain a low temperature during the transport. A thin styrofoam sheed separated the abalone from the ice packs during the approximately 8 h air-freight to the laboratory in Hobart. Abalone used in this study for enzyme activity and movement/$\dot{M}O_2$ experiments were 12 and 15 months old, respectively.

Acclimation to environmental conditions

At the laboratory, abalone were left to recover from transport for at least seven days. Mixed groups of 13 to 18 individuals of each abalone type were housed in four separate aquaria (height: 20.0 cm, diameter: 70.5 cm, water volume: 56.2 L). Recirculating seawater (700 L; 16 to 17 °C) was bio-filtered, UV-treated, vigorously aerated, and changed by 50% every two days. Nitrogenous waste levels were measured daily with an accuracy of 0.25 mg L^{-1} for ammonia and nitrite levels and an accuracy of 5 mg L^{-1} for nitrate levels (API® Saltwater Master Kit Test, Australia). Ammonia and nitrite levels did not reach 0.5 mg L^{-1} and nitrate levels remained under 25 mg L^{-1}. Animals were fed with a commercial food pellet *ad libitum* (Halo, Skretting, Australia) and kept in constant darkness to simulate aquaculture conditions. Faeces and uneaten food were removed from the aquaria every morning. Temperature (16 to 17 °C), oxygen level (91 to 99% O_2sat), and salinity (31.3 to 34.4) were monitored daily with a portable digital thermometer (HQ10, Hach, USA), digital oxygen meter (HQ10, Hach, USA), and digital salinity meter (HQ14, Hach, USA), respectively.

Seven days post transport, two aquaria were warmed to 23 °C at a rate of 1.5 °C day^{-1}. Additionally, the dissolved oxygen level in one of the two aquaria per temperature (16 and 23 °C) was lowered to 70% O_2sat (± 2% O_2sat) at a rate of 4% O_2sat h^{-1} using regulated nitrogen injection via solenoid valves (Atlantic, OxyGuard, Denmark). The abalone were maintained under these conditions (either 16 or 23 °C at either 70 or 100% O_2sat) for 2 to 3 weeks, which is sufficient for warm-temperature acclimation in abalone (Dahlhoff and Somero, 1993). The control temperature of 16 °C was chosen because this temperature is close to the preferred temperature of 17 and 19 °C for *H. rubra* and *H. laevigata*, respectively, and further, abalone are conditioned at 16 °C on-farm before live shipment (Gilroy and Edwards, 1998; A. Krsinich, JTA, pers. com., 2014). The 23 °C temperature was selected as a treatment because it is the average summer temperature that abalone experience

on-farm at JTA (A. Krsinich, JTA, pers. comm., 2014). Similarly, 100% O_2sat was used as the control treatment and 70% was chosen as a treatment because this oxygen level is towards the lowest level commonly experienced by abalones held at JTA (Wassnig *et al.*, 2009; Wassnig *et al.*, 2010; Alter, unpubl. data). Water quality, light exposure, and feeding practices were maintained as described above.

Movement and oxygen consumption rate experiments

Experimental set-up

Abalone were starved for 12 h prior to the commencement of movement and $\dot{M}O_2$ experiments. Immediately prior to the experiments, the shells of eight abalone (mixture of pure species and hybrids) were brushed and blotted dry to minimize possible micro-organisms. Then, the animals were transferred to individual respiration chambers at their respective acclimation condition (16 or 23 °C; 70 or 100% O_2sat). The abalone could move freely within the chambers. Two chambers without abalone were included in each experimental run to account for any microbial respiration. The respiration chambers were made from PVC pipes with screw caps and glass bottoms (volume: 743 ml, diameter: 16 cm, height: 3.7 cm) which were placed in a water bath (total volume 520 L). A small pump (3 L min^{-1}, Aquapro tabletop feature pump, Australia) inside each chamber maintained water movement at all times to ensure homogenous oxygen levels throughout the chamber. Wires from oxygen and temperature optodes (FirestingO2, Pyroscience, Germany) were hermetically sealed into the screw caps using two cable glands. Temperature was sampled (FirestingO2, Pyroscience, Germany) at a rate of 1 s^{-1} in the common water bath and in one of the 10 respiration chambers per experimental run. Oxygen level was sampled (FirestingO2, Pyroscience, Germany) at a rate of 1 s^{-1} in each respiration chamber. The oxygen optodes were calibrated in air-saturated seawater for 100% O_2sat and in sodium sulphite-saturated seawater for 0% O_2sat. Temperature was maintained in the water bath using aquarium heaters (Weipro, titanium heaters). Oxygen level was maintained by constantly bubbling the water with air for the 100% O_2sat treatment or with regulated bursts of nitrogen for the 70% O_2sat treatment (Atlantic, OxyGuard, Denmark). The abalone were kept in constant darkness with only infrared lighting beneath the water bath to enable photographs to be taken for movement analysis. Two cameras (GoPro Hero 3, USA) installed 30 cm beneath the water bath captured photographs of four chambers, each at a rate of 6 min^{-1}.

Movement and $\dot{M}O_2$ were immediately monitored after the abalone were introduced into the chamber. The chambers were sealed hermetically until the oxygen levels dropped by a maximum of 5% O_2sat (maximum of 40 min). Subsequently, the chambers were flushed with water from the water bath to replenish the oxygen levels back to test levels (70% or 100% O_2sat, approximately 3 min). This cycle was repeated for ~ 65 h after which the oxygen levels were raised to 100% O_2sat for all individuals including those acclimated to 70% O_2sat and closed hermetically until the oxygen levels reached 0 to 5% O_2sat to determine P_{crit}. The experiments were terminated and abalone were removed from the chambers, rinsed with Milli-Q® water and whole animal wet weight (WW), tissue WW and shell length (SL) were determined. Wet weight was determined using a digital balance with an accuracy of 0.001 g and size was measured using a digital caliper with a precision of 0.01 mm.

Analyses

Animal movement, temperature, and oxygen level were measured constantly throughout each 60 to 65 h trial. The initial 12 to 17 h were omitted from the analysis in order to remove the impact of handling on movement and $\dot{M}O_2$.

Animal movement was determined for each abalone in each experiment using the images taken every 10 s with the cameras beneath the water bath. Every tenth image per abalone was converted into an avi file. Individual animal movement (in SL [cm] h^{-1}) was then calculated by manually tracking the shortest distance between the position of the mouth of each individual between every frame (100 s) using the plugin MTrackJ for Image J (FIJI) (Abramoff *et al*., 2004; Myrick, 2009; Schindelin *et al*., 2015). Movement was determined in 3 to 9 individuals for each type of abalone and treatment during chronic exposure and in all individuals during P_{crit} determinations (n = 9 to 12 for each type of abalone and treatment; Table 5.1).

Routine $\dot{M}O_2$ (in µmol g WW^{-1} h^{-1}) was calculated calculated from the linear decrease in % O_2sat measured in each respiration chamber during a sealed respirometry cycle across a 10 min recording according to Eq. 1:

$$\dot{M}O_2 = \frac{\Delta FO_2}{\Delta t} \times (P_B - P_S) \times \beta_{O_2} \times Vol \times 0.2093 \qquad (1),$$

where ΔFO_2 is the difference of the fractional oxygen concentrations, Δt is the difference in time (s), P_B is the barometric pressure (kPa), P_S is the saturation vapor pressure of water

(kPa), β_{O_2} is the capacitance of water for oxygen, *Vol* is the chamber volume minus the abalone volume (L, assuming 1 g wet mass equals 1 ml), and 0.2093 is the fractional concentration of oxygen in water. Routine $\dot{M}O_2$ during the final 48 h of the chronic exposure was determined for 16 °C and 100% O_2sat-acclimated individuals (n = 7 to 9 for each type of abalone) and for all treatments during P_{crit} experiments. Critical oxygen levels were determined by least squares regression, i.e. fitting a broken-line function to the routine $\dot{M}O_2$ data of each individual (Muggeo, 2008).

Resting $\dot{M}O_2$ (in µmol g WW^{-1} h^{-1}) was calculated in order to gain insight into the effect of increasing oxygen levels for 70% O_2sat-acclimated individuals immediately before commencement of P_{crit} experiments. Resting $\dot{M}O_2$ was determined by calculating the lowest 10% of measured $\dot{M}O_2$ values. Animal movement data were compared to $\dot{M}O_2$ data and individuals were inactive during the lowest 10% of routine $\dot{M}O_2$. Resting $\dot{M}O_2$ was determined for all treatments during P_{crit} experiments and in addition for 70% O_2sat-acclimated abalone during chronic exposure. It was assumed that resting $\dot{M}O_2$ of 100% O_2sat-acclimated animals was not different during chronic exposure and during P_{crit} experiments at oxygen levels above P_{crit}. Q_{10} values of resting and routine $\dot{M}O_2$ were calculated according to Eq. 2:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}} \qquad (2),$$

where R_2 is the rate ($\dot{M}O_2$) at high temperature (T_2, 23°C) and R_1 is the rate at low temperature (T_1, 16°C).

Enzyme activity determination

Following two weeks under acclimation conditions, 10 to 12 individual hybrids, *H. rubra* and *H. laevigata* were starved for three days. Then, the animals were removed from the tank with a blunt spatula and rinsed with Milli-Q® water. Whole animal WW, tissue WW, and SL were measured and the animals were subsequently dissected (time lapsed during this procedure ~ 3 min). Muscle tissue (foot and adductor muscles combined) was immediately frozen in liquid nitrogen. Foot and adductor muscles were combined to reach a sufficient amount of tissue to conduct the enzyme analyses. The tissues were stored at -80 °C until used in lactate dehydrogenase (LDH) and tauropine dehydrogenase (TDH) enzyme assays. Deep frozen muscle tissue (approximately 1 g) was mixed with ice-cold Tris-HCl buffer (75 mmol L^{-1}

Tris-HCl, 1 mmol L^{-1} EDTA and 8 mmol L^{-1} $MgCl_2$, pH 7.8) at a ratio of 1:2 w:v (weight : volume) and homogenized in a tissue lyser (Tissue Lyser II, Qiagen). The homogenates were centrifuged (12000 g for 6 min at 4 °C) and the supernatant was subdivided into two parts for determinations of TDH and LDH activities. Supernatants were stored at -80 °C until analysed.

Enzyme assays were carried out in 96 well plates with changes in absorbance measured at 340 nm (SpectraMax 190 Microplate reader). The assays were conducted in a temperature controlled room at either 16 or 23 °C according to the acclimation condition of each individual. Reaction mixtures for both LDH and TDH assays contained imidazole-HCl buffer (50 mmol L^{-1}, pH 7.0). Lactate dehydrogenase assays also contained pyruvate (2.5 mmol L^{-1}) and NADH (0.15 mmol L^{-1}). Tauropine dehydrogenase assays also contained taurine (80 mmol L^{-1}). Measurements were carried out in triplicates and controls without substrate (pyruvate for LDH and taurine for TDH) were run to correct for non-specific activity. Protein concentration in the muscle tissue was determined spectrophotometrically according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Statistical analyses

Statistical tests were performed using SigmaPlot 13.0. Normality was tested with Shapiro–Wilk tests, and homogeneity of variance was tested with Brown–Forsythe tests. Probability of less than 0.05 was considered as significant. Paired t-tests were conducted to test for differences in size and weight between types of abalone used for enzyme activity determinations and movement/MO_2 experiments. The influence of acclimation, activity, and type of abalone on a particular test variable was tested using two-way analyses of variances (ANOVA) with Student-Newman-Keuls test (SNK) for multiple pairwise comparisons. The variables tested were: total distance travelled during chronic exposure, average distance travelled above and below P_{crit}, resting $\dot{M}O_2$ during chronic exposure and P_{crit} experiments, routine $\dot{M}O_2$ during P_{crit} experiments (log transformed to satisfy normality assumptions), and TDH and LDH activity. Normality assumptions were not satisfied for data of P_{crit} values and a non-parametric Scheirer-Ray Hare two-way ANOVA was conducted (Scheirer *et al*., 1976; Sokal and Rohlf, 1995).

To test if abalone express a circadian rhythm in movement and/or routine $\dot{M}O_2$, one-way repeated measures (RM) analyses of variances (ANOVA) were conducted using movement and routine $\dot{M}O_2$ data of each type of abalone from the control chronic exposure treatment

(16 °C and 100% O_2sat). Movement data were not normal and a non-parametric Friedman one-way RM ANOVA on ranks was conducted. Normality was satisfied for routine $\dot{M}O_2$ data and a one-way RM ANOVA was used. To test if average distances travelled were different above and below P_{crit}, paired t-tests were conducted within each type of abalone at each treatment.

Table 5.1: Critical oxygen levels (P_{crit}) [% O_2sat] and number of individuals (*n*) of hybrids, *H. rubra* and *H. laevigata* acclimated to different environmental conditions. Different *lower case letters* in superscript indicate significant differences between acclimation conditions ($p < 0.05$, non-parametric Scheirer-Ray Hare, SNK). Mean ± SE.

	Hybrid		***H. rubra***		***H. laevigata***	
	P_{crit} [% O_2sat]	***n***	**P_{crit} [% O_2sat]**	***n***	**P_{crit} [% O_2sat]**	***n***
16 °C 100% O_2sat	38.1 ± 2.9^{a}	11	37.1 ± 3.7^{a}	11	40.2 ± 1.9	9
16 °C 70% O_2sat	48.7 ± 5.0^{b}	11	47.1 ± 5.1^{ab}	11	49.7 ± 3.9	12
23 °C 100% O_2sat	48.2 ± 2.5^{b}	11	45.8 ± 2.8^{ab}	12	45.5 ± 1.8	12
23 °C 70% O_2sat	48.2 ± 2.0^{b}	12	48.4 ± 2.2^{b}	13	48.7 ± 3.8	10

Results

Abalone size

Abalone used for movement and $\dot{M}O_2$ experiments were 15 months old. Total wet weight (WW) and shell length (SL) of hybrids was similar in comparison to *H. laevigata* ($p > 0.05$) and 1.2-fold larger in comparison to *H. rubra* ($p < 0.001$, t-test; Table 5.2). Individuals used for enzyme activities were 12 months old and differed in WW and SL. Hybrids were slightly larger (1.1- to 1.3-fold) in comparison to both pure species ($p < 0.01$) and *H. laevigata* was slightly larger (1.1-fold) in comparison to *H. rubra* ($p < 0.05$, t-test; Table 5.2).

Table 5.2: Wet mass [g], shell length [cm] and number of hybrids, *H. rubra* and *H. laevigata* used for enzyme activity determination (aged 12 months) and movement/oxygen consumption rate ($\dot{M}O_2$) experiments (aged 15 months). Different superscript *lower case letters* indicate significant differences between type of abalone ($p < 0.05$, t-test). Mean ± SE.

Experiment		**Hybrids**	***H. rubra***	***H. laevigata***
Enzyme	**Wet mass [g]**	3.8 ± 0.2^{a}	2.8 ± 0.1^{b}	3.2 ± 0.1^{c}
	Shell length [cm]	3.14 ± 0.04^{a}	2.72 ± 0.03^{b}	3.02 ± 0.04^{c}
	Number individuals	44	42	36
Movement/$\dot{M}O_2$	**Wet mass [g]**	13.4 ± 0.6^{a}	10.4 ± 0.4^{b}	12.1 ± 0.4^{a}
	Shell length [cm]	4.68 ± 0.07^{a}	4.16 ± 0.06^{b}	4.71 ± 0.05^{a}
	Number individuals	45	48	48

Movement

Movement during chronic exposure

No circadian rhythm in movement was observed in any of the three types of abalone when exposed to control conditions. Individual distances travelled (in SL h^{-1}) were highly variable throughout time of day and average distances travelled were not statistically different across time of day in hybrids and both pure species ($p > 0.05$, non-paramteric one-way RM ANOVA on ranks; Fig. 5.1A). Since no difference between day and night was observed in the control treatment, other treatments (16 °C and 70% O_2sat, 23 °C and 100% O_2sat, 23 °C and 70% O_2sat) were not analysed.

There was a significant effect of acclimation condition ($p < 0.01$) and type of abalone ($p < 0.001$) on the movement activity whereas the interaction of the two factors was not significant ($p > 0.05$) (two-way ANOVA; Fig. 5.2). Across all acclimation conditions, hybrid movement was 1.5 to 2.5-fold lower than *H. rubra* movement, however, post-hoc tests revealed that the difference was only significant at 16 °C and 70% O_2sat ($p < 0.05$; Fig. 5.2). Hybrids moved similar distances to *H. laevigata* at 23 °C at both oxygen levels ($p > 0.05$) and 1.4-fold longer numerical distances at 16 °C at both oxygen levels, yet this difference was not statistically significant ($p > 0.05$; Fig. 5.2).

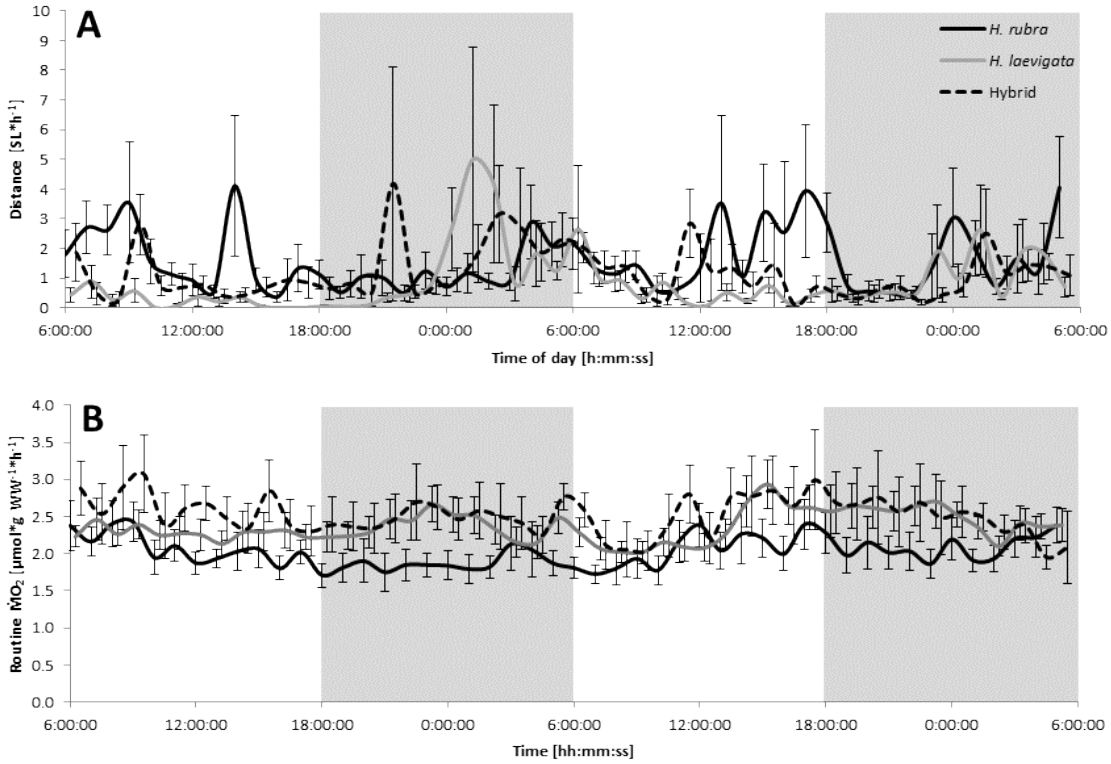


Fig. 5.1: Distance travelled per hour [SL h^{-1}] (A) and routine oxygen consumption rate ($\dot{M}O_2$) [µmol g WW^{-1} h^{-1}] (B) of *H. rubra* (black line), *H. laevigata* (grey line), and hybrid abalone (dashed line) during two days at 16 °C and 100% air saturation. Mean ± SE; n = 5 to 9 (for A); n = 7 to 9 (for B). Grey backgrounds indicate night in Hobart, Australia, but abalone were held in constant darkness also during day. Data are offset between types of abalone for visual clarity of error bars.

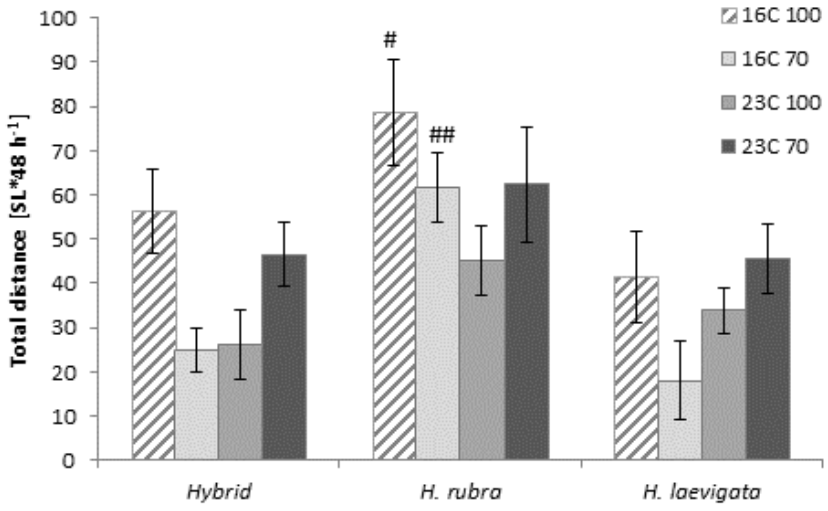


Fig. 5.2: Total distance travelled (shell lengths [SL 48 h^{-1}]) across a two day observation period of hybrids, *H. rubra*, and *H. laevigata*. Abalone were acclimated and exposed to different environmental conditions (see legend). # = significantly different to *H. laevigata*. ## = significantly different to hybrids and *H. laevigata* ($p < 0.05$, two-way ANOVA, SNK). Mean ± SE; number of individuals stated in Table 5.1.

Movement during acutely decreasing oxygen level

Mean distance travelled [SL h^{-1}] by hybrids was higher at oxygen levels below P_{crit} in comparison to above P_{crit} (Fig. 5.3A). When acclimated to control conditions, hybrid movement was 2.5-fold lower above than below P_{crit} ($p < 0.007$, t-test). This trend was also observed for all other conditions, yet it was not significant for hybrids at 16 °C and 70% O_2sat ($p > 0.05$, t-test). A similar pattern was seen in *H. laevigata* (Fig. 5.3C). In contrast, *H. rubra* movement was similar above and below P_{crit} except for 23 °C and 100% O_2sat-acclimated individuals for which movement doubled below P_{crit} ($p < 0.001$, t-test; Fig. 5.3B).

Both, acclimation condition ($p = 0.02$) and type of abalone ($p < 0.001$) affected the movement activity above P_{crit} whereas the interaction of the two factors was not significant ($p > 0.05$) (two-way ANOVA). Hybrid movement above P_{crit} was similar between acclimation conditions and averaged 1.68 ± 0.19 SL h^{-1} ($p > 0.05$). This was different to both pure species. Movement of *H. rubra* above P_{crit} at control conditions was 1.7- to 1.9-fold lower than movement at 70% O_2sat at both 16 °C ($p < 0.05$) and 23 °C ($p < 0.006$). Movement above P_{crit} for *H. laevigata* acclimated to 16 °C and 70% O_2sat was 3- and 4-fold lower in comparison to 23 °C and 100% O_2sat ($p < 0.05$) and 23 °C and 70% O_2sat, respectively ($p < 0.02$). This lead to differences in mean distance travelled above P_{crit} between types of abalone. Hybrids moved similar distances to pure species when acclimated to control oxygen level at both temperatures ($p > 0.05$; Fig. 5.3A-C). Yet, when acclimated to the lower oxygen level, *H. laevigata* moved 6-fold shorter distances at 16 °C (0.41 ± 0.07 SL h^{-1}; $p < 0.001$) and *H. rubra* moved 2-fold longer distances at 23 °C (4.28 ± 0.65 SL h^{-1}; $p = 0.01$) in comparison to hybrids.

Movement of abalone below P_{crit} was affected by acclimation condition ($p < 0.001$) but not by type of abalone ($p > 0.05$, two-way ANOVA; Fig. 5.3A-C). The interaction of the two factors was not significant ($p > 0.05$, two-way ANOVA). Below P_{crit}, abalone moved half the numerical distance at 16 °C (2.49 ± 0.16 SL h^{-1}) in comparison to 23 °C (5.07 ± 0.33 SL h^{-1}; $p < 0.001$; Fig. 5.3A-C). Oxygen acclimation had no influence on this result ($p > 0.05$; Fig. 5.3A-C).

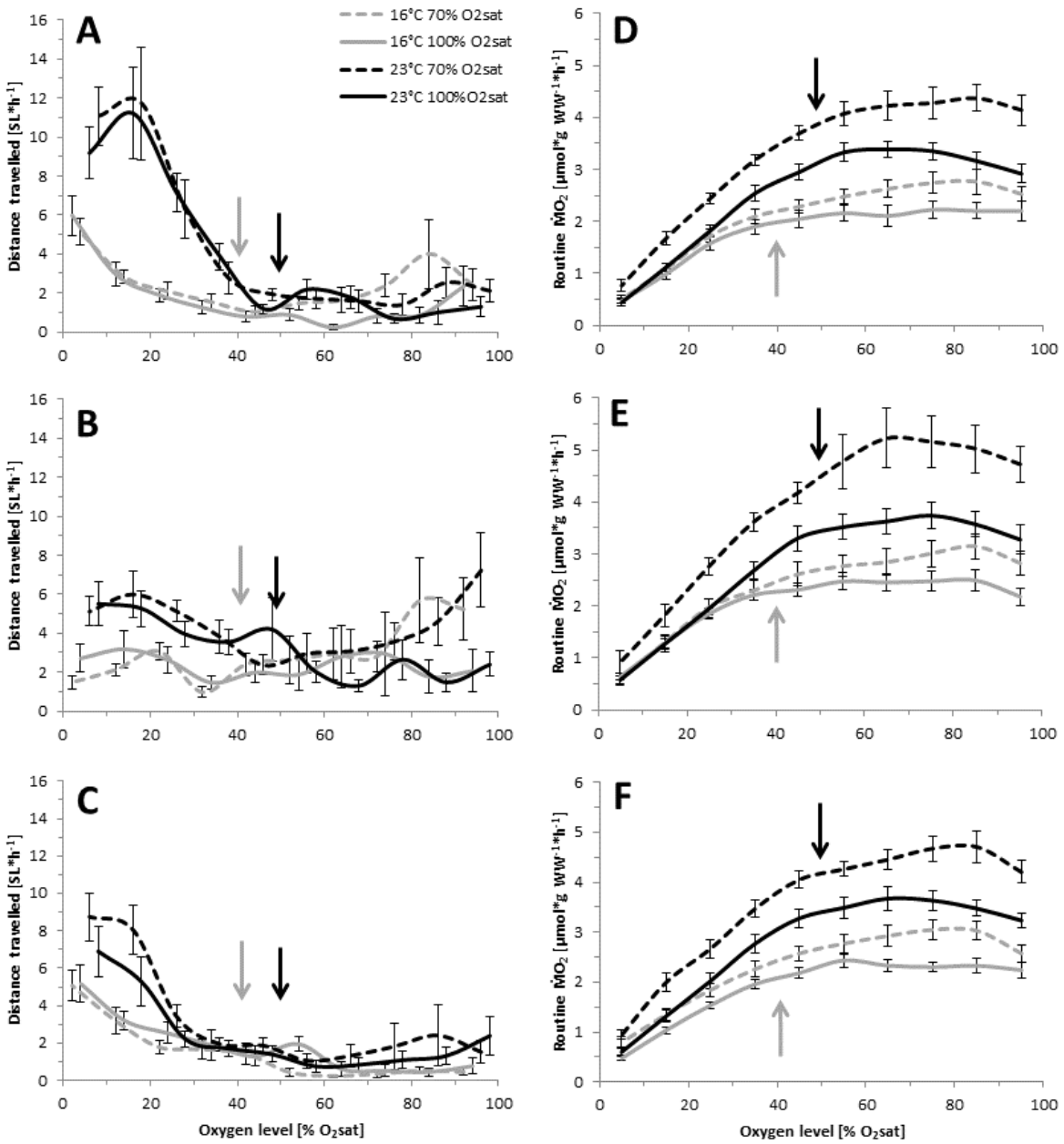


Fig. 5.3: Movement [SL h^{-1}] (A–C) and routine $\dot{M}O_2$ [µmol g WW^{-1} h^{-1}] (D–F) over decreasing dissolved oxygen levels from 100% to 5% for the hybrid (A, D), *H. rubra* (B, E), and *H. laevigata* (C, F). Abalone were acclimated to 16 °C (grey lines) and 23 °C (black lines) at 70% O_2sat (dashed lines) and 100% O_2sat (solid lines). Grey arrows indicate P_{crit} for acclimation condition 16 °C and 100% O_2sat. Black arrows indicate P_{crit} at all other tested acclimation conditions. Movement data are offset for visual clarity of error bars. Mean ± SE; number of individuals in Table 5.1.

Energetics

Routine and resting oxygen consumption rate

No circadian rhythm was observed for routine $\dot{M}O_2$ (in µmol g WW^{-1} h^{-1}) in any of the three types of abalone during the two day observation period at control conditions ($p > 0.05$, one-way RM ANOVA; Fig. 5.1B), thus, other treatments were not analysed.

Acclimation condition significantly affected routine $\dot{M}O_2$ above P_{crit} ($p < 0.001$), but type of abalone ($p > 0.05$) and the interaction of the two factors was not significant ($p > 0.05$) (two-way ANOVA; Fig. 5.3D-F). For hybrids and both pure species, routine $\dot{M}O_2$ was 1.5-fold higher at 23 °C in comparison to 16 °C ($p < 0.001$) and 1.3-fold higher at 70% O_2sat in comparison to 100% O_2sat at 16 °C ($p < 0.001$) and 23 °C ($p < 0.001$; Fig. 5.3D-F).

Resting $\dot{M}O_2$ (in µmol g WW^{-1} h^{-1}) above P_{crit} showed a similar trend to routine $\dot{M}O_2$ for hybrids and both pure species (Fig. 5.4). Yet, effects of acclimation condition ($p < 0.001$) and type of abalone ($p < 0.02$) on resting $\dot{M}O_2$ above P_{crit} were significant. The interaction of the two factors was not significant ($p > 0.05$, two-way ANOVA; Fig. 5.4). Resting $\dot{M}O_2$ for 70% O_2sat-acclimated hybrids was 1.3-fold higher than resting $\dot{M}O_2$ of 100% O_2sat acclimated hybrids during P_{crit} experiments at both temperatures ($p < 0.05$). Yet, this was not observed during chronic exposure when 70% O_2sat-acclimated hybrids had similar resting $\dot{M}O_2$ to 100% O_2sat acclimated hybrids at both temperatures ($p > 0.05$; Fig. 5.4). These results were also observed for *H. laevigata* at both temperatures, but only at 16 °C for *H. rubra* (Fig. 5.4). At the higher temperature, resting $\dot{M}O_2$ of 70% O_2sat-acclimated *H. rubra* during chronic exposure (2.49 ± 0.20 µmol g WW^{-1} h^{-1}) was 1.3-fold lower in comparison to resting $\dot{M}O_2$ of 100% O_2sat-acclimated *H. rubra* (3.29 ± 0.24 µmol g WW^{-1} h^{-1}) ($p = 0.01$; Fig. 5.4). Comparisons between types of abalone showed that hybrids had a lower resting $\dot{M}O_2$ at 16 °C and 70% O_2sat during chronic exposure (1.79 ± 0.10 µmol g WW^{-1} h^{-1}) in comparison to *H. laevigata* (2.37 ± 0.16 µmol g WW^{-1} h^{-1}) ($p < 0.05$; Fig. 5.4). Q_{10} values for resting $\dot{M}O_2$ are stated in Table 5.3.

Table 5.3: Q_{10} values (between 16 and 23 °C) for resting oxygen consumption rates ($\dot{M}O_2$) of *H. rubra*, *H. laevigata*, and hybrids acclimated to 70% and 100% O_2sat. $\dot{M}O_2$ above P_{crit}= $\dot{M}O_2$ was determined during critical oxygen level (P_{crit}) experiments at oxygen levels above P_{crit}. Oxygen was raised to 100% O_2sat also for 70%-acclimated animals immediately before P_{crit} experiments commenced. $\dot{M}O_2$ chronic exposure= $\dot{M}O_2$ was determined during chronic exposure to 70% O_2sat.

	Acclimation	**Hybrid**	***H. rubra***	***H. laevigata***
$\dot{M}O_2$ above P_{crit}	100% O_2sat	2.04	1.76	1.86
	70% O_2sat	1.97	1.62	1.83
$\dot{M}O_2$ chronic exposure	70% O_2sat	2.35	1.23	1.78

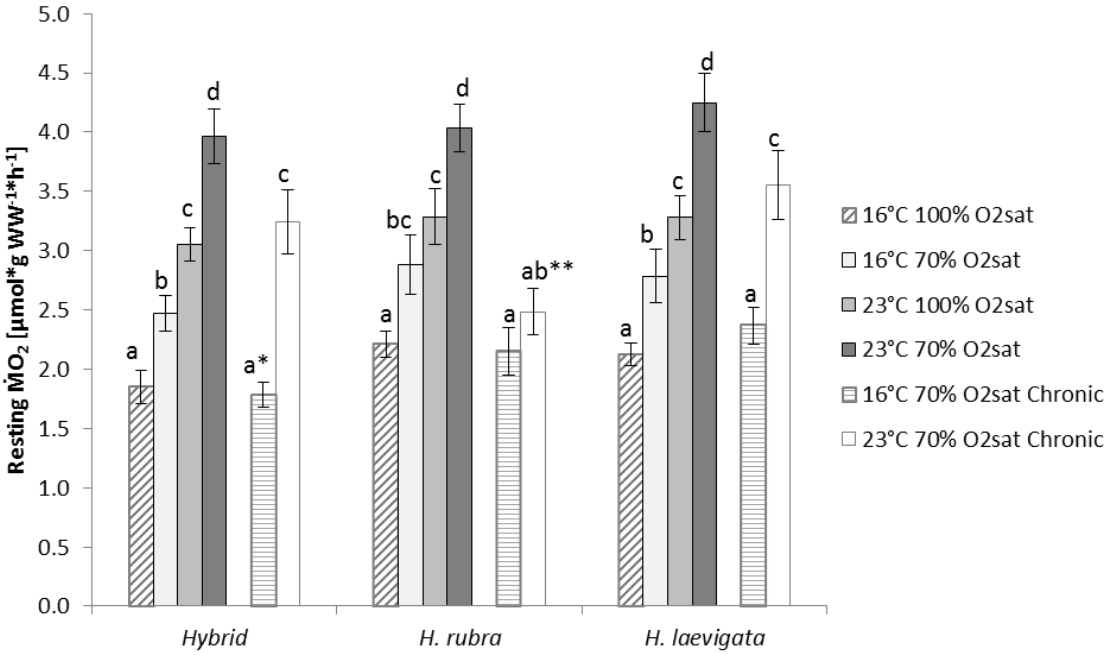


Fig. 5.4: Resting oxygen consumption rate ($\dot{M}O_2$) [µmol g WW^{-1} h^{-1}] (A) of hybrid, *H. rubra*, and *H. laevigata* abalone above critical oxygen levels (P_{crit}) and during chronic exposure (Chronic). Different *lower case letters* = significant differences between treatments within given type of abalone. * = significantly lower resting $\dot{M}O_2$ in hybrids in comparison to *H. laevigata* at the given treatment. ** = significantly lower resting $\dot{M}O_2$ in *H. rubra* in comparison to hybrids and *H. laevigata* at the given treatment ($p < 0.05$, two-way ANOVA, SNK). Mean ± SE; n = 10 to 12.

P_{crit} values

Critical oxygen levels of routine $\dot{M}O_2$ were significantly affected by acclimation condition ($p < 0.01$) but type of abalone ($p > 0.05$) and the interaction of the two factors was not significant ($p > 0.05$) (non-parametric two-way ANOVA; Table 5.1). Critical oxygen levels of routine $\dot{M}O_2$ of abalone were lower by 10% O_2sat under the control conditions when compared to all other conditions (38.3 ± 2.9% O_2sat *versus* 47.8 ± 1.1% O_2sat, respectively; $p = 0.001$; Table 5.1).

Enzyme activities during chronic exposure

There was a significant effect of acclimation condition on TDH activity (in U mg P^{-1}) ($p < 0.001$), but type of abalone ($p > 0.05$) and the interaction of the two factors was not significant ($p > 0.05$) (two-way ANOVA; Fig. 5.5A). For hybrids, TDH activity was 1.2- to 1.4-fold higher at the lower oxygen level than at the control oxygen level ($p < 0.05$) and was 2.1- to 2.5-fold higher at the higher temperature in comparison to the control temperature ($p < 0.001$; Fig. 5.5A). Similar results were observed for both pure species ($p > 0.05$; Fig. 5.5A).

Similar to TDH activity, also LDH activity was influenced by acclimation condition ($p < 0.001$), but not by type of abalone ($p > 0.05$, two-way ANOVA; Fig. 5.5B). The interaction of the two factors was also not significant ($p > 0.05$, two-way ANOVA; Fig. 5.5B). Hybrids and both pure species LDH activity doubled at the higher temperature in comparison to control temperature ($p < 0.001$; Fig. 5.5B). Acclimation oxygen level had no influence on LDH activity in any of the three types of abalone acclimated to 16 °C ($p > 0.05$; Fig. 5.5B). In contrast, LDH activity at 23 °C was higher under lower oxygen level than at control oxygen level ($p > 0.05$; Fig. 5.5B).

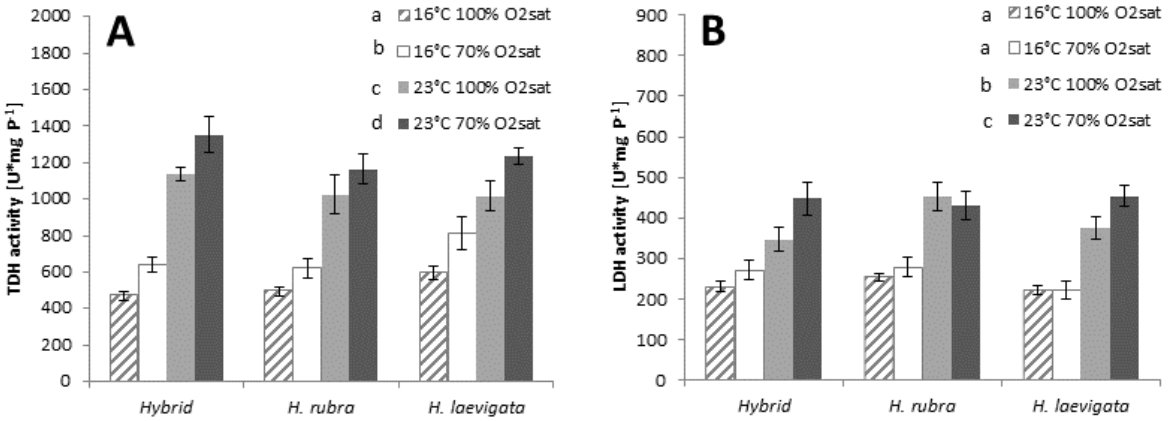


Fig. 5.5: Enzyme activities [U mg P^{-1}] of tauropine dehydrogenase (TDH) (A) and lactate dehydrogenase (LDH) (B) in the foot and adductor muscles (combined) of hybrids, *H. rubra*, and *H. laevigata* acclimated to different environmental conditions. Note the difference in the y-axis scale. Different *lower case letters* = significant differences between acclimation conditions ($p < 0.05$, two-way ANOVA, SNK). Mean ± SE; n = 7 to 12.

Discussion

In this study it was addressed whether hybrids differ in behaviour and the use of aerobic and anaerobic energy production in comparison to parental pure species. Results were indicative but provided little support for the hypothesis that hybrids have an energetic advantage that may contribute to their growth heterosis.

The lack of any clear differences in movement in this study may be a result of several generations of domestication and selection for commercial performance under the farm conditions. Indeed, it has been demonstrated that changes in behaviour can be among the first adaptations during domestication processes (Huntingford, 2004; Lachambre *et al*., 2017). As a result, captive *H. tuberculata*, for example, moved less than their wild counterparts (Peck *et al*., 1987; Lachambre *et al*., 2017). In agreement, differences in movement between *H. rubra* and *H. laevigata* were not significantly different in the present study, while their wild counterparts have been reported to noticeably differ in movement patterns (Shepherd, 1973). Abalone mainly move because of disturbance or in order to find food (Denny, 1980; Donovan and Carefoot, 1997; Robinson *et al*., 2013; Buss *et al*., 2015). For example, cultured *H. laevigata* increased movement under a restricted food ratio in comparison to an unlimited ratio (Buss *et al*., 2015). Abalone use chemosensory cues to detect food (Allen *et al*., 2006). The absence of food in the present study may have reduced movement activity due to the absence of chemical cues. The intermediate movement behaviour of hybrids, as reported from

aquaculture farmers, may become clearer in the presence of chemical cues, over longer observation periods, and/or in less restrictive environments such as aquaculture grow-out tanks. Long-term observations of movement patterns and growth rates under commercial aquaculture practices could shed light on these suggestions.

In the present study, abalone were held in constant darkness to simulate conditions that abalone are exposed to on the farm where they were sourced from. At JTA, the grow-out tanks are housed in rooms that are dark for 24 h (A. Krsinich, JTA, pers. comm., November 2013). It is possible that the chosen light regime reduced movement of abalone. Aquaculture farmers report less movement of abalone under darkness which is in agreement with results from the present study and previous reports. In the present study, hybrids and *H. laevigata* moved approximately 65 and 80 cm day^{-1}, respectively. Yet, during exposure to a diurnal cycle, hybrids and *H. laevigata* moved 50- and 29- fold longer numerical distances, respectively (at 22 °C, formulated diet provided, Currie *et al*., 2016). The constant darkness in the present study may have also prevented the detection of a circadian rhythm. In the present study, individual distances travelled were highly variable throughout time of day and average distances travelled were not different across time of day in hybrids, *H. rubra*, and *H. laevigata*. Yet, when exposed to a diurnal cycle, *H. laevigata* and hybrids moved little during the day and predominantly during night (Currie *et al*., 2016). Similarly, also *H. diversicolor supertexta* exhibited a circadian rhythm when exposed to a natural diurnal cycle but the rhythm was not observed when individuals were exposed to continuous light (Jan *et al*., 1981). Thus, by exposing abalone to constant darkness, abalone farmers may indeed achieve reduced movement which may promote increased growth rates in case food is abundant.

Movement of all abalone types tended to decrease with increasing temperature at 100% O_2sat, yet, anaerobic enzyme activities were 2- to 3-fold higher at 23 °C in comparison to 16 °C. This suggests that abalone movement at higher temperatures is more energetically costly. In accordance, energy expenditure for movement in *H. kamtschatkana* is twice as high in summer compared to winter (Donovan and Carefoot, 1998). While TDH predominantly supports anaerobic energy production during functional hypoxia (e.g. crawling), LDH is the predominant enzyme during environmental hypoxia (Gäde, 1988; Baldwin *et al*., 1992). Abalone at 16 °C tended to move more at control oxygen level, compared with the lower oxygen level while the opposite trend was seen at 23 °C. Yet, in comparison to control oxygen level, exposure to lower oxygen level resulted in increased TDH activities at both temperatures and increased LDH activities at 23 °C. Increased anaerobic enzyme activity

indicates that locomotion may require more anaerobic energy at reduced oxygen levels. In addition, exposure to 70% O_2sat at higher temperature may be suboptimal and abalone increasingly support their aerobic metabolism with anaerobic energy even at oxygen levels above their P_{crit}. It is noteworthy, that in a study addressing growth rates of *H. laevigata*, 1 year old individuals had higher biomass gain at 22 °C in comparison to biomass gain at 14 and 18 °C. Yet, two year old *H. laevigata* showed no difference in biomass gain at 18 and 22 °C, suggesting age-specific thermal optima for growth (Stone *et al*., 2013). The study on growth was conducted at normoxia. It may be possible that a reduction in oxygen levels pose a higher constrain to the metabolism of abalone than an increase in temperature within the range of tested temperatures within this study.

Movement pattern differences between hybrids and pure species were observed during P_{crit} determinations (Fig. 5.3). Oxygen levels were raised to 100% O_2sat for the 70% O_2sat-acclimated abalone immediately before P_{crit} experiments commenced. In response to the change in oxygen level, movement of *H. rubra* increased, movement of *H. laevigata* decreased, and movement of hybrids did not change. The lack of behavioural response from hybrids suggests that they are less sensitive to acute changes in oxygen levels which may be advantagous in the aquaculture environment. On some aquaculture farms, oxygen levels commonly decrease to approximately 70% O_2sat at the end of the slab tank and the direction of the water flow is changed every two weeks (Wassnig *et al.*, 2009; Wassnig *et al.*, 2010; A. Krisnich, JTA, pers. comm., 2014). Hence, cultured abalone regularly experience acute changes in oxygen levels. A lower sensitivity of hybrids to these acute changes may partly reason their improved growth. Oxygen sensitivity is energetically costly due to macromolecule damage and repair and/or replacement of those molecules (Hawkins and Day, 1999; Somero, 2002). Hence, a lower sensitivity to environmental factors can lead to an energetic advantage which may be the case for hybrids. In accordance, lower sensitivity to environmental change in hybrids in comparison to *H. rubra* and *H. laevigata* has been demonstrated during acute temperature increase at various oxygen conditions (Chapter 6).

Hybrids showed the most pronounced movement increase at oxygen levels below P_{crit} which may indicate that hybrids, in comparison to pure species, had a greater capacity for escaping unfavourable oxygen conditions. In contrast, movement of *H. rubra* was similar above and below P_{crit} at 16 °C which could be associated with the higher movement in this species above P_{crit} and during chronic exposure in comparison to hybrids and *H. laevigata* (Fig. 5.2 and 5.3). The abalone were starved for three days before the P_{crit} experiments commenced

and potentially the higher movement in *H. rubra* may have resulted in increased exhaustion of energy reserves which may have suppressed a flight response below P_{crit}. In general, energy storage is low in abalone and glycogen concentrations within the digestive gland fully deplete within five days of fasting in *H. kamtschatkana* (Carefoot *et al*., 1993). At 23 °C, however, movement was increased below P_{crit} also in *H. rubra*, but the maximum distance travelled was only half of that observed for hybrids and 30% lower than that of *H. laevigata*. Increased movement below P_{crit} at 23 °C in comparison to 16 °C in all three types of abalone suggests that the motivation to find more suitable oxygen conditions is enhanced at higher temperatures. From an aquaculture perspective, these results indicate that growth rates of abalone could be enhanced, especially during summer months, by ensuring that oxygen levels remain above the P_{crit} of abalone. Critical oxygen tensions of abalone examined in this study were between 38 and 47% O_2sat, yet older *H. rubra* × *H. laevigata* hybrids with two years of age and four year old *H. laevigata* had a P_{crit} of ~ 80% O_2sat (A. Morash, Mount Allison University, unpubl. data, 2014; Harris *et al*., 1999). On aquaculure frams, abalone are commonly exposed to 70% O_2sat (Wassnig *et al.*, 2009; Wassnig *et al.*, 2010). Hence, control of oxygen in grow-out tanks may lead to improved growth in older individuals only.

During chronic exposure, resting $\dot{M}O_2$ was similar between 70 and 100% O_2sat-acclimated hybrids and *H. laevigata*, while during P_{crit} experiments, resting $\dot{M}O_2$ increased in all three types of abalone acclimated to 70% O_2sat in comparison to 100% O_2sat-acclimated abalone. It is likely that this is a compensatory response to the increase in oxygen levels prior to the commencement of the P_{crit} experiment; an oxygen debt payback. This compensatory response is in accordance with increased enzyme activities at 70% O_2sat and further supports the suggestion that abalone supplement their aerobic metabolism with anaerobic energy even at oxygen levels above their P_{crit}. In contrast to hybrids and *H. laevigata*, resting $\dot{M}O_2$ of *H. rubra* during chronic exposure to 23 °C and 70% O_2sat was lower than resting $\dot{M}O_2$ of *H. rubra* acclimated and exposed to 100% O_2sat. This observation suggests that *H. rubra* went into metabolic depression at the higher temperature and the lower oxygen level. Similarly, in a temperature challenge experiment, the oxygen debt payback in *H. rubra* lasted for five hours after oxygen levels were increased from 70% to 100% O_2sat (Chapter 6). Oxygen consumption rates of hybrids and *H. laevigata* also increased immediately after the change in oxygen level occurred. Yet, $\dot{M}O_2$ returned to control values after one hour suggesting a lower impact of oxygen on $\dot{M}O_2$ in hybrids and *H. laevigata* than *H. rubra* (Chapter 6).

There are two contrasting hypotheses for how metabolic rate leads to improved growth rates, i.e. the compensation hypothesis and the increased intake hypothesis (reviewed in Burton *et al*. (2011)). In the present study, the hybrid abalone had generally lower resting $\dot{M}O_2$ than their pure parental species. Combined with the reported higher growth of hybrids on-farm, the tendency for lower $\dot{M}O_2$ of hybrids may give support for the compensation hypothesis. This would further support the above suggestion that hybrids have reduced maintenance costs (repair and replacement of oxygen damaged macromolecules) due to lower oxygen sensitivity. Determining repair and/or degradation proteins, for example ubiquitin, could improve the understanding of maintenance costs between types of abalone.

In summary, the differences in movement, anaerobic enzyme activities, and $\dot{M}O_2$ between hybrids and pure species in this study are indicative but not conclusive in supporting the original hypothesis that hybrids have an energetic advantage over pure species. Longer observation periods and examining older animals (where size differences are more pronounced) could potentially reveal that trends observed in the present study may indeed lead to growth advantages of the hybrid.

CHAPTER 6: Hybrid vigour in abalone

All of the research contained within this chapter is in press for publication in *Aquaculture* as:

Alter K, Andrewartha SJ, Morash AJ, Clark TD, Hellicar AD, Leon R, Elliott NG (2017) Hybrid abalone are more robust to multi-stressor environments than pure parental species. *Aquaculture* **478:** 25-34.

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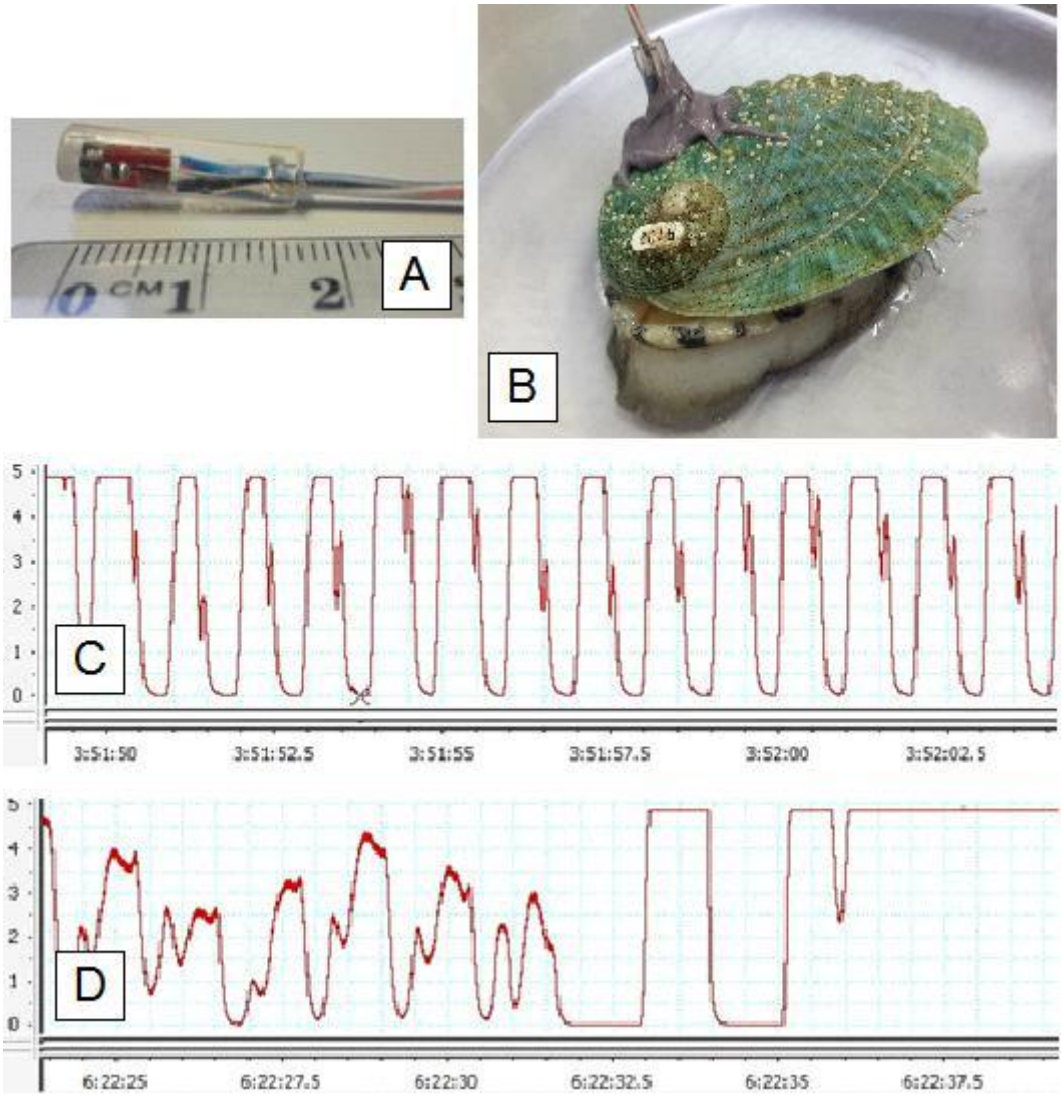

In this chapter, abalone heart rate was measured with a custom built biosensor (A) from the University of Tasmania. After the sensor was placed above the abalone heart (B), heart rate was measured at various environmental conditions. Heart rate traces were rhythmic during exposure to non-stressful conditions (C) and became arrhythmic during exposure to stressful conditions (D).

Hybrid abalone are more robust to multi-stressor environments than pure parental species

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Abstract

Many hybrids of marine molluscs show improved growth in comparison to their pure parental species. Yet, little is known about the physiological mechanisms underlying the better hybrid performance. This study determined movement, oxygen consumption rate ($\dot{M}O_2$), and heart rate of 22 month old cultured abalone *H. rubra, H. laevigata,* and their interspecies hybrid, the latter of which exhibits improved growth rate. Abalone were exposed to an acute temperature increase following acclimation to 16 or 23 °C at high and low oxygen levels (100% or 70% air saturation, respectively). Hybrid and *H. laevigata* movement was generally not affected by temperature and oxygen levels, yet *H. rubra* showed a strong thermal response. Heart rate and $\dot{M}O_2$/temperature slopes revealed that hybrids were least affected by oxygen levels. Arrhenius break-point temperatures of hybrids and *H. laevigata*, but not *H. rubra*, were generally higher when abalone were acclimated to 23 °C in comparison to 16 °C. The hybrid had more stable maximum heart rate and $\dot{M}O_2$ values across acclimation conditions in comparison to *H. laevigata* and *H. rubra*. Thus, it appears that the hybrid is able to maintain physiological functions over a broader environmental range. This improved tolerance to environmental fluctuations may bolster energy metabolism and improve growth in variable environments.

Keywords: Hybrid vigour, abalone, heart rate, oxygen consumption rate, movement

Introduction

Heterosis or hybrid vigour occurs when an interspecies individual shows improved fitness or superior phenotypic traits in comparison to both parental pure species. A positive heterosis for growth has been reported for many shellfish species, including oysters, scallops, and abalone (Guo, 2009). Yet, the physiological mechanisms underlying superior performance of hybrids are not well understood. It has been suggested that positive heterosis for growth rate may be caused by differences in metabolic efficiency, which can be measured through whole-animal metabolism (Toro *et al*., 1996).

The metabolic rate and ultimately the growth rate of marine ectotherms are strongly influenced by the environmental conditions to which individuals are exposed. Temperature and oxygen are the major driving factors. Oxygen is required in the aerobic pathways that produce the energy needed to drive all cellular reactions (Dahlhoff and Somero, 1993; Grieshaber *et al*., 1993). During exposure to hypoxia, species that are adapted to this condition, undergo metabolic suppression which results in reduced oxygen consumption rates ($\dot{M}O_2$) (Storey and Storey, 1990). In contrast, species that are not adapted to hypoxia modify physiological functions to increase oxygen uptake, for example by increasing their heart stroke volume amongst others (Ragg and Taylor, 2006a). The rate of oxygen consumption increases with temperature throughout the ecological range resulting in increased heart rate to transport the required oxygen to the cells where it is needed. As a result, $\dot{M}O_2$ and heart rate typically correlate across ecologically relevant temperatures. This correlation, however, is finite and $\dot{M}O_2$ and heart rate will reach a maximum at a critical temperature above which animals can no longer function (Dahlhoff and Somero, 1993). This upper critical temperature can be calculated in Arrhenius plots (log of physiological rate against temperature) and is also known as the Arrhenius break-point temperature (Dahlhoff and Somero, 1993). Thus, $\dot{M}O_2$ and heart rates can give valuable insight into the metabolism and thermal sensitivity of an animal (Marshall and McQuaid, 1992; Dahlhoff and Somero, 1993; Chen *et al*., 2016).

Thermal sensitivities are not always fixed and species with the capacity for acclimation may shift the temperature where their physiological rates are at a maximum across their ecological temperature range (Gabriel and Lynch, 1992; Dahlhoff and Somero, 1993). As such, acclimation can enhance fitness by ensuring that the optimal performance temperature overlaps with the temperatures prevailing in the environment (Gabriel and Lynch, 1992; Seebacher *et al*., 2010). Determining thermal sensitivities of $\dot{M}O_2$ and heart rate in hybrids and parental pure species under a range of acclimation conditions could provide insight into

the improved growth reported for many hybrids. For example, a lower metabolic sensitivity to environmental factors in hybrids may translate into lower long-term metabolic costs and higher abiotic tolerances as seen in comparisons between gastropod species from contrasting environmental habitats (Hawkins, 1995; Somero, 2002; Sokolova and Pörtner, 2003).

Traditionally, maximum temperatures are determined by exposing an animal to gradually increasing temperatures and measuring the temperature where an animal loses balance and mobility (Cowles and Bogert, 1944). This method provides limited additional information on the current physiological knowledge regarding improved performance of hybrids. Yet, understanding movement patterns and overall activity during warming may reveal a behavioural response by hybrids which results in a physiological advantage over pure species. Activity in gastropods indirectly affects fitness because of the high costs associated with locomotion and mucous production (Denny, 1980; Donovan and Carefoot, 1997). Lower activity in hybrids, for example, may result in an energetic advantage that could translate into faster growth.

Abalone are a marine mollusc (Werner *et al*., 1995; Cenni *et al*., 2009), and populations of the blacklip abalone *H. rubra* and the greenlip abalone *H. laevigata* occur on shallow reefs along the southern coasts of Australia (Shepherd, 1973). Their distribution ranges overlap, however *H. rubra* is found further south in most common water temperatures of 11 to 19 ºC compared with most common temperatures of 12 to 23 ºC for *H. laevigata* (Shepherd, 1973). The two species typically differ in their microhabitat, whereby *H. rubra* inhabits sheltered areas of rocks while *H. laevigata* is often found on rocks exposed to rough waters. Additionally, the species show distinct behavioural differences, whereby *H. rubra* often wanders nocturnally to search for food, while *H. laevigata* can remain in one place for several years (Shepherd, 1973).

Naturally occurring hybrids between *H. rubra* and *H. laevigata* are rare (Brown and Murray, 1992; Brown, 1995) but it has become a key land-based aquaculture product in Australia because of its growth advantage (Li, 2008; Guo, 2009; Hamilton *et al*., 2009). The behaviour of the hybrid is also better suited for culture because it does not escape grow-out tanks unlike its parental species *H. rubra* (Guo, 2009). Nevertheless, maximal growth may still be impaired because of fluctuating environmental conditions prevailing on abalone farms (Chapter 2). On most abalone farms, water temperature varies with the local environmental conditions and on some abalone farms oxygen levels frequently decrease to 70% O_2sat within grow-out tanks (Wassnig *et al.*, 2009; Wassnig *et al.*, 2010; Alter, unpubl. data).

This study measured physiological and behavioural responses of cultured *H. rubra*, *H. laevigata*, and their interspecies hybrid to an acute increase in temperature following acclimation to 16 or 23 °C and to high (100% O_2sat) or low (70% O_2sat) oxygen levels. The aim was to determine if differences in $\dot{M}O_2$, heart rate, and movement patterns of hybrids could help explain their growth heterosis relative to the pure parental species. It was hypothesised that hybrid abalone have a more consistent response to temperature, a higher thermal tolerance, and are less sensitive to changes in oxygen level than their parental pure species.

Methods

Transport and acclimation in the laboratory

Individually tagged (Hallprint tags) *H. rubra* (n = 72), *H. laevigata* (n = 75), and their interspecies hybrid (n = 72, female *H. rubra* × male *H. laevigata*) were obtained from Jade Tiger Abalone (JTA) in Indented Head, Victoria, Australia. Individuals were sourced from one family to minimise effects of genetic variation. Abalone were age matched (22 months old) and differed significantly in total WW ($p < 0.001$ for all types of abalone; t-test) and SL ($p < 0.001$ for hybrids *versus* pure species, pure species not different to each other $p > 0.05$; t-test) (hybrid: 36.0 ± 0.8 g WW and 67.4 ± 0.6 mm SL, *H. rubra*: 30.0 ± 0.9 g WW and 61.4 ± 0.6 mm SL, *H. laevigata* 25.8 ± 0.7 g WW and 61.4 ± 0.5 mm SL). Abalone were transferred to the laboratory at CSIRO in Hobart, Tasmania, according to standard industry shipping practices. In brief, animals were levered off the substrate in the grow-out tanks with a blunt spatula and transferred to purge tanks with a constant temperature of 16 °C and no food supply. After two days the abalone were transferred into plastic bags filled with oxygen at 300% O_2sat and placed in a styrofoam box. The box was equipped with ice packs to maintain a low temperature during the transport. A thin styrofoam sheet separated the abalone from the ice packs during the approximately 8 h air-freight to the laboratory in Hobart.

At the CSIRO laboratories, the abalone were housed in mixed groups in four separate aquaria (height: 20.0 cm, diameter 70.5 cm, water volume: 56.2 L), with 13 to 18 individuals of *H. rubra*, *H. laevigata*, and their interspecies hybrid in each. This allowed adjustment of later experimental conditions (see below) without requiring subsequent relocation of individuals. Recirculating seawater (700 L; 16 to 17 °C) was bio-filtered, UV-treated, vigorously aerated, and changed by 50% every two days. Nitrogenous waste levels were measured daily with an accuracy of 0.25 mg L^{-1} for ammonia and nitrite levels and an accuracy of 5 mg L^{-1} for nitrate

levels (API® Saltwater Master Kit Test, Australia). Ammonia and nitrite levels did not reach 0.5 mg L^{-1} and nitrate levels remained under 25 mg L^{-1} except on two occasions when a measurement of 40 mg L^{-1} was recorded. Animals were fed with a commercial food pellet *ad libitum* and kept in constant darkness (as on commercial farm) to reduce disturbance caused by light. Faeces and uneaten food were removed from the aquaria every morning. Temperature (16 to 17 °C), oxygen level (91 to 99% O_2sat), and salinity (31.3 to 34.4) were monitored daily with a portable digital thermometer (HQ10, Hach, USA), digital oxygen meter (HQ10, Hach, USA), and digital salinity meter (HQ14, Hach, USA), respectively. Abalone were given seven days to recover from transport prior to being exposed to acclimation conditions.

Subsequently, two aquaria with individuals of *H. rubra*, *H. laevigata*, and their interspecies hybrid were warmed to 23 °C at a rate of 1.5 °C day^{-1}. Additionally, the oxygen levels in one of the two aquaria per temperature (16 and 23 °C) were lowered to 70% O_2sat (± 2% O_2sat) at a rate of 4% O_2sat h^{-1} using regulated nitrogen injection via solenoid valves (Atlantic, OxyGuard, Denmark). Abalone were held under these conditions (either 16 or 23 °C at each of 70 and 100% O_2sat) for two weeks, which has been reported to be sufficient for warm-temperature acclimation in abalone (Dahlhoff and Somero, 1993). The respective oxygen contents of 70 and 100% O_2sat were 6.9 and 9.8 mg L^{-1} at 16 °C and 6.0 and 8.6 mg L^{-1} at 23 °C. The 16 °C treatment served as a control group because this matched the culture temperature at JTA before transfer from the farm to the laboratory. The 23 °C temperature was selected as a treatment because it represents the highest temperature that abalone commonly experience at the farm during summer (A. Krsinich, JTA, pers. comm., November 2014). The control oxygen condition was 100% O_2sat, while 70% O_2sat was chosen because this oxygen level is towards the lowest level commonly experienced by abalone held at JTA (Wassnig *et al.*, 2009; Wassnig *et al.*, 2010; Alter, unpubl. data). Further, previous studies have shown that prolonged exposure of *H. laevigata* to oxygen levels lower than 70% O_2sat at 18 °C caused increased mortality (Harris *et al.*, 1999). Water quality assessment, light exposure, and feeding practices were maintained as described above.

Animal preparation and experimental design

In the morning of each trial, the shells of seven abalone (mixture of hybrids and pure species) were brushed and blotted dry to minimise the presence of micro-organisms which may contribute to metabolic measurements. A small < 5 mm hole was drilled with a Dremel in the

shell above the heart. Care was taken to not damage the underlying pericardium during drilling and visual inspection of the heart confirmed that the tissue of all abalone used in experiments remained intact. A custom made biosensor (CSIRO and the University of Tasmania, Hellicar *et al*., 2015, see below) was attached flush with the shell using impregum (3M ESPE Impregum Soft). The animals were then transferred to individual respiration chambers at their respective acclimation conditions (16 or 23 °C; 70 or 100% O_2sat). Respiration chambers were PVC pipes with screw caps and glass bottoms (volume 1.2 L, height 6 cm, diameter 16 cm; 8 per trial) and were large enough for the abalone to move freely. Also the wires from the biosensor were long enough to not influence locomotion of the abalone. Each respirometer was equipped with a submersible pump (3 L min^{-1}, Aquapro tabletop feature pump) to ensure that the water remained well mixed. Two cable glands in the screw caps enabled the chambers to hermetically seal around the wires from the biosensor and an optical oxygen probe (FirestingO2, Pyroscience, Germany). The chambers were placed in a water bath (total volume 520 L) with a recirculating water supply. In the water bath, temperature was maintained using aquarium heaters (Weipro, titanium heater) and dissolved oxygen level was maintained by constantly bubbling water with air for the 100% O_2sat treatment or with regulated bursts of nitrogen for the 70% O_2sat treatment (Atlantic, OxyGuard, Denmark). The abalone were left to recover for 20 to 22 h without food supply. Pre-experiments had shown that this time frame is sufficient for heart rate and $\dot{M}O_2$ of *H. rubra*, *H. laevigata*, and their hybrid to return to resting rates. During this initial recovery period, the chambers were intermittently sealed hermetically for a maximum of 18 min per cycle, during which oxygen levels dropped by a maximum of 5% O_2sat. Subsequently, the chambers were flushed with water from the water bath for 4.5 min to replenish oxygen levels back to test levels (70% or 100% O_2sat). The experimental set-up was placed in a temperature controlled room. The room was dark except for visible red lighting beneath the chambers. The red lighting enabled video recording with two cameras (GoPro Hero 3, USA) that were installed 30 cm below the water bath. Each camera captured four respiration chambers throughout the temperature challenge trials (see below).

Temperature challenge trials commenced 20 to 22 h after the abalone were placed in their individual respirometry chambers. These trials were conducted at the respective oxygen levels (70% or 100% O_2sat). In addition, abalone acclimated to 70% O_2sat were also tested at 100% O_2sat. For this oxygen condition (subsequently referred to as 70/100% O_2sat), the oxygen levels were acutely (within 20 min) raised to 100% immediately before experiments

commenced. During the thermal challenge, intermittent flow cycles were maintained as described for the initial recovery period. The cycles were upheld for 50 min after which temperatures were raised stepwise by 2 °C during a 15 min time interval and the protocol was repeated. This stepwise process continued until 31 °C was reached. The experiments were terminated after the abalone had been exposed to 31 °C for 50 min. Animal movement, oxygen levels, heart rate, and water temperature were constantly measured throughout the experiments (see below). The abalone were removed from the chambers, rinsed with Milli-Q® water and individual whole animal, tissue and shell WW as well as SL were determined. Wet mass was determined using a digital balance with an accuracy of 0.001 g and size was measured using a digital calliper with a precision of 0.01 mm. Between 9 to 12 individuals were tested per experimental treatment for each pure species and the interspecies hybrid.

Analyses

The cameras beneath the water bath took images every 10 s throughout the temperature challenge trials. Every tenth image from each experimental run were converted into an avi file. Individual animal movement (in SL [cm] h^{-1}) was then calculated by manually tracking the shortest distance between the position of the mouth of each individual between every frame (100 s) using the plugin MTrackJ for Image J (FIJI) (Abramoff *et al*., 2004; Myrick, 2009; Schindelin *et al*., 2015). The 15 min time interval between each fixed experimental temperature (during which temperature was raised by 2 °C) was included in the movement analyses because individuals showed high activity during or immediately after this interval. Movement during the 15 min interval was added to the higher temperature that was reached after the interval.

Oxygen level was measured with an optical oxygen probe (FirestingO2, Pyroscience, Germany) at a sampling rate of 1 Hz in each of the eight respiration chambers (seven containing abalone plus one blank). For each experimental run, one chamber without an abalone was included to serve as a control to account for background respiration by any micro-organisms in the seawater and on the surfaces of the respiration chambers. The optical oxygen probes were calibrated in air-saturated seawater for 100% O_2sat and in sodium sulphite-saturated seawater for 0% O_2sat. Temperature was measured (FirestingO2, Pyroscience, Germany) at a sampling rate of 1 Hz in the common water bath and in one of the eight respiration chambers per experimental run. Oxygen consumption rate was determined for each individual abalone after 20 min exposure to each temperature. Blanks

and individual $\dot{M}O_2$ (in µmol g WW^{-1} h^{-1}) were calculated from the linear decrease in % O_2sat measured in each respiration chamber during a sealed respirometry cycle across a 10 min recording according to Eq. 1 (see Chapter 5).

The wired biosensor that was fitted in the shell of each abalone allowed *in situ* real-time measurement of heart rate. An LED illuminated the heart with light which was then reflected and detected by a sensor unit, interfaced through PowerLab (ADInstruments, Australia), and recorded using the software LabChart 7 (ADInstruments, Australia). The signal from the heart was measured at a sampling rate of 400 Hz for 5 min and was repeatedly cycled through the seven respiration chambers during the temperature challenge. Heart rate (in bpm) was then calculated by splitting the 5 min heart rate signal for each individual at each temperature into multiple 10 s sequences stepped at 5 s (Hellicar *et al*., 2015). Each sequence was analysed using the autocorrelation method described in Hellicar *et al*. (2015). Median values across all sequences were used to estimate the entire heart rate sequence.

Upper critical temperatures of $\dot{M}O_2$ and heart rate were determined via Arrhenius break-point temperature (ABT) plots. For this, the log of $\dot{M}O_2$ and heart rate were calculated and plotted against temperature in 1000 K^{-1} (Fig. 6.1). The Arrhenius break-point temperature and corresponding heart rate and $\dot{M}O_2$ maxima were then determined by least squares regression (i.e. fitting a broken-line function to the data of each individual) (Muggeo, 2008).

Q_{10} values of 16 °C-acclimated abalone were calculated using $\dot{M}O_2$ and heart rate data of each individual at 17 and 25 °C according to Eq. 3.

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}} \qquad (3),$$

where R_2 is the rate ($\dot{M}O_2$ or heart rate) at high temperature (T_2, 25°C) and R_1 is the rate at low temperature (T_1, 17°C).

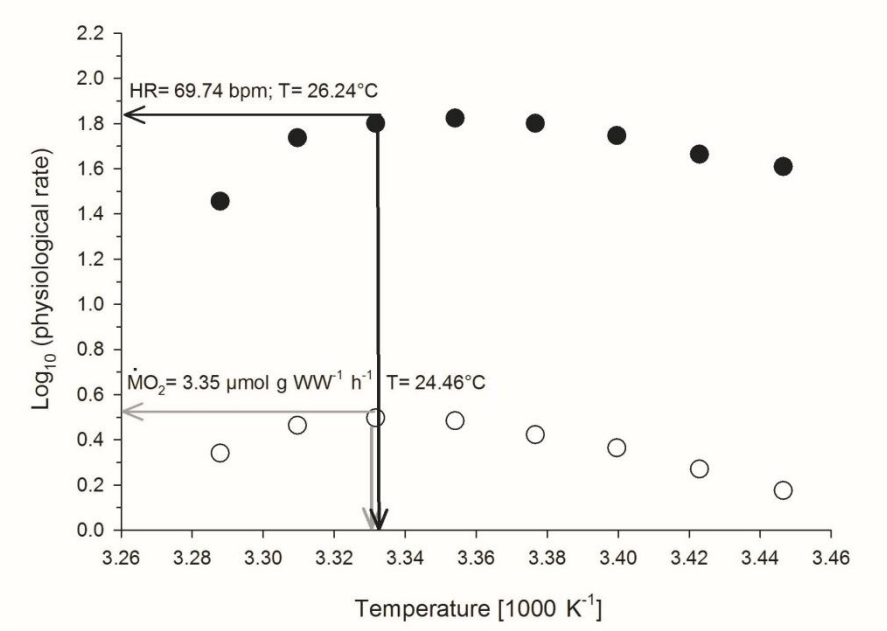

Fig. 6.1: Arrhenius break-point temperatures and corresponding physiological rates (heart rate [bpm], open circles; oxygen consumption rate ($\dot{M}O_2$) [µmol g WW^{-1} h^{-1}], closed circles) of one representative hybrid acclimated to 16 °C and 100% O_2sat. Arrhenius break-point temperatures (vertical arrows) and corresponding (horizontal arrows) heart rate (grey) and $\dot{M}O_2$ (black) maxima were determined by fitting a broken-line function to the data.

Statistical analyses

Pearson's correlation coefficients were calculated for $\dot{M}O_2$ and heart rate data from 17 to 25 °C in the abalone groups acclimated to 16 °C to determine if the two parameters correlate. Pearson's correlation coefficients were calculated using Microsoft Excel 2010. A significance of $p < 0.05$ was selected for this and all following statistical analyses. Statistical tests to compare movement, slopes and intercepts of heart rate and $\dot{M}O_2$/temperature relationships, and ABTs and corresponding maximum heart rate and $\dot{M}O_2$ values were performed with R v. 3.3.0 (R Core Team, 2016) using the R package *nlme* (Pinheiro *et al.*, 2016). Pairwise multiple tests were carried out using the R package *lsmeans* and Bonferroni adjustments were applied to reduce the probability of false positives (type I error) (Lenth, 2016).

Movement, ABTs and corresponding heart rate and $\dot{M}O_2$ maxima were compared between treatments and between types of abalone using generalised least squares (GLS) models. Variances of heterogeneity were accounted for in the coefficient estimation of the model. For movement data, a first-order autoregressive structure was assumed to deal with repeated measures and significant autocorrelation.

The effect of increasing temperature on heart rate and $\dot{M}O_2$ of 16 °C-acclimated abalone was tested with an ANCOVA. Linear mixed-effects models were fitted with abalone individuals as a random effect to take into account that measures of heart rate and $\dot{M}O_2$ were taken repeatedly over time. This model also accounted for heteroscedasticity and significant autocorrelation. For heart rate data the mass of the individuals was used to weight the model fitting, taking into account the natural variations in heart rate occurring because of mass differences.

Results

For clarity throughout the results, acclimation conditions are stated in subscript following the type of abalone e.g. *H. laevigata*$_{16C\_70/100}$ refers to *H. laevigata* acclimated to 16 °C and 70% O_2sat and measured at 100% O_2sat. Subscripts without oxygen level are given in cases when no specification between oxygen acclimation is needed for clarification.

Movement

Experimental temperature had little influence on hybrid abalone movement, which was similar to *H. laevigata* but different to *H. rubra* (Fig. 6.2). Hybrid$_{16C}$ moved similar distances irrespective of experimental temperature or oxygen level (on average 1.01 ± 0.20 SL h^{-1}) (Fig. 6.2A). Yet, hybrid$_{23C}$ had a fluctuating movement pattern and travelled a 1 to 3-fold longer distance at 25 and 29 °C in comparison to 23 and 27 °C. Yet, this pattern was only statistically significant between 23 and 25 °C as well as 25 and 27 °C in hybrid$_{23C_100}$ ($p < 0.005$) and hybrid$_{23C_70/100}$ ($p < 0.05$) but not in hybrid$_{23C_70}$ ($p > 0.05$) (Fig. 6.2D). Similarly, *H. laevigata*$_{16C}$ movement remained constant during temperature increase in all oxygen groups and averaged 0.43 ± 0.09 SL h^{-1} (Fig. 6.2C). *Haliotis laevigata*$_{23C\_70}$ had a fluctuating movement pattern in that distances travelled were three times longer at 25 °C in comparison to 23 °C ($p < 0.05$) and 27 °C ($p < 0.05$) (Fig. 6.2F). Movement of *H. laevigata*$_{23C\_70/100}$ averaged 1.21 ± 0.30 SL h^{-1} at 25, 27, and 29 °C, and was 33-fold higher than movement at 23 °C ($p < 0.02$) (Fig. 6.2F). *H. laevigata* movement was generally similar to hybrids. Hybrid$_{16C}$ moved on average twice the numerical distance of *H. laevigata*$_{16C}$, but this trend was not statistically significant ($p > 0.05$; Fig. 6.2A and C). Significantly longer distances were only observed for hybrid$_{23C_100}$ in comparison to *H. laevigata*$_{23C\_100}$ at 25 °C

($p < 0.05$) and 31 °C ($p < 0.05$) and for hybrid$_{23C_70/100}$ in comparison to *H. laevigata*$_{23C\_70/100}$ at 23 °C ($p < 0.05$, Fig. 6.2D and F).

In contrast to hybrids and *H. laevigata*, *H. rubra* showed a strong movement response with increasing temperature (Fig. 6.2B and E). For *H. rubra*$_{16C}$, irrespective of oxygen level, movement increased significantly with temperature from 17 to 27 °C ($p < 0.05$) and decreased from 27 to 31 °C ($p < 0.05$) (Fig. 6.2B). Average distances moved near the acclimation temperature of 17 °C were 7 and 8 times lower for *H. rubra*$_{16C\_70/100}$ (0.13 ± 0.09 SL h^{-1}) in comparison to *H. rubra*$_{16C\_100}$ (0.88 ± 0.44 SL h^{-1}) and *H. rubra*$_{16C\_70}$ (1.09 ± 0.41 SL h^{-1}), respectively, but the differences were not statistically significant ($p > 0.05$). At other temperatures, *H. rubra*$_{16C}$ movement was also similar between oxygen conditions and peaked at 27 °C with an average of 2.84 ± 0.45 SL h^{-1} (Fig. 6.2B). A similar trend was seen for *H. rubra*$_{23C\_100}$ with significantly lower movement at the acclimation temperature of 23 °C (0.52 ± 0.18 SL h^{-1}) and peak movement at 29°C (1.81 ± 0.40 SL h^{-1}, $p < 0.02$). In contrast, movement of *H. rubra*$_{23C\_70}$ and *H. rubra*$_{23C\_70/100}$ was similar across temperatures with average distances of 0.84 ± 0.30 SL h^{-1} and 0.77 ± 0.25 SL h^{-1}, respectively (Fig. 6.2E). In comparison to *H. rubra*$_{16C\_100}$ and *H. rubra*$_{16C\_70}$, hybrids at the same acclimation condition moved shorter distances at 21 °C ($p < 0.05$), 25 °C ($p < 0.02$), and 27 °C ($p < 0.05$; Fig. 6.2A and B). For hybrid$_{16C_70/100}$ and *H. rubra*$_{16C\_70/100}$ the difference was only significant at 27 °C ($p < 0.01$; Fig. 6.2A and B). Few statistical differences were observed between hybrid$_{23C}$ and *H. rubra*$_{23C}$. At 27°C, *H. rubra*$_{23C\_100}$ moved 3-fold longer distances ($p < 0.02$) and at 25 °C, *H. rubra*$_{23C\_70/100}$ moved 2-fold shorter distances ($p < 0.05$) in comparison to hybrids at the same acclimation condition (Fig. 6.2D and E).

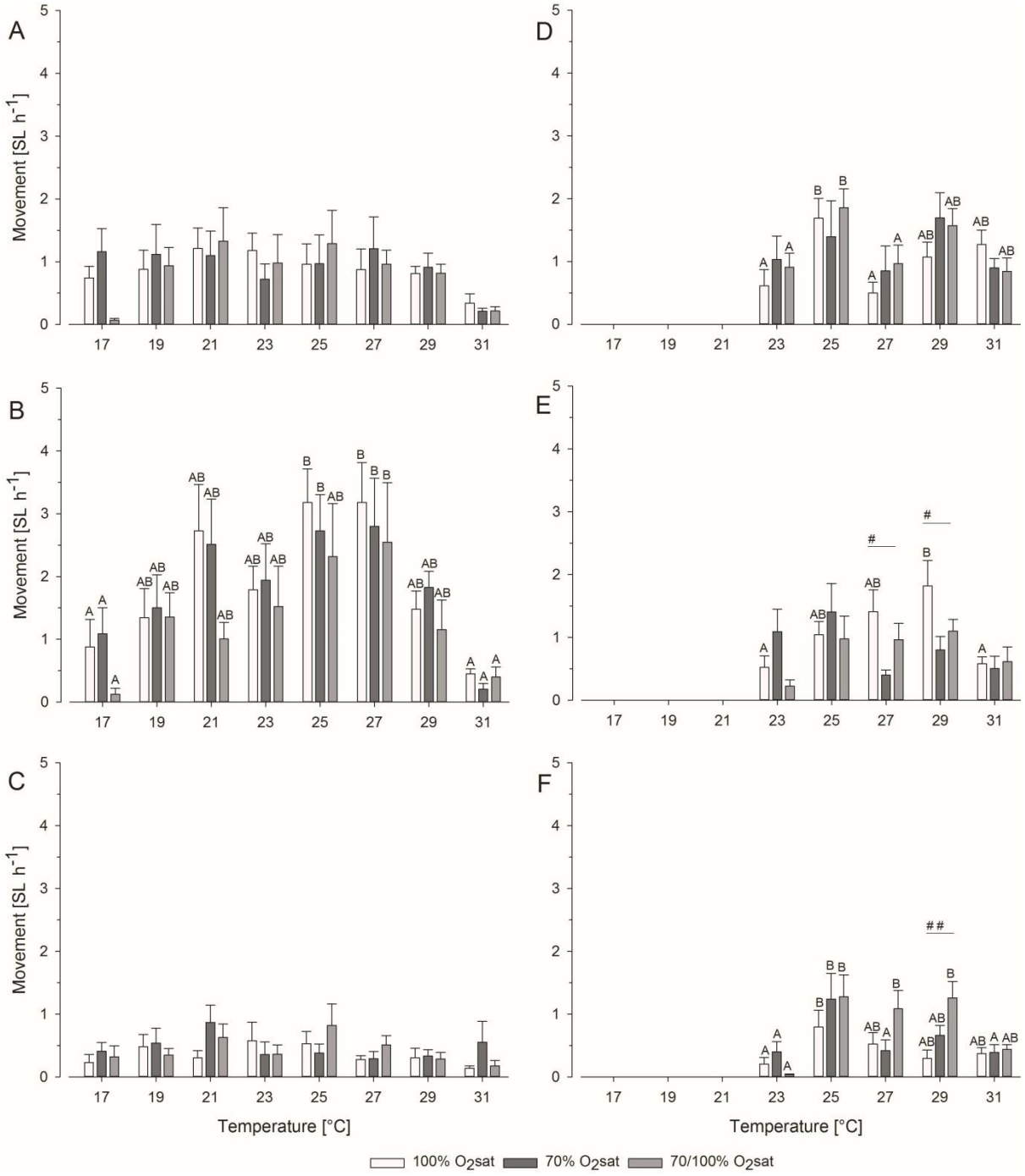

Fig. 6.2: Distance moved (shell length (SL) [cm] h^{-1}) at experimental temperatures between 17 and 31 °C of hybrid abalone (A, D), *H. rubra* (B, E), and *H. laevigata* (C, F) acclimated to 16 °C (A–C) and 23 °C (D–F) at 100% O_2sat (open bars), 70% O_2sat (dark grey bars) and 70% O_2sat but measured in 100% O_2sat (light grey bars). Different *upper case letters* indicate significant differences between temperatures within given acclimation condition. Within a given temperatures, # indicates significant differences between 100% and 70% O_2sat acclimation and ## indicates significant differences between 100% and 70/100% O_2sat (abalone acclimated to 70% O_2sat but measured in 100% O_2sat) acclimation. Mean ± SE. n = 9 to 12.

Physiological rate increments with temperature ramping

Heart rate and $\dot{M}O_2$ of hybrids and both pure species in all acclimation groups responded with a similar pattern to experimental temperature (example in Fig. 6.1). Indeed, $\dot{M}O_2$ was positively correlated with heart rate in all acclimation groups of hybrid$_{16C}$ as well as both pure species$_{16C}$ (Pearson's correlation coefficient, $p < 0.01$) (Table 6.1). Both physiological parameters increased with increasing temperatures up to a temperature between 25 and 29 °C and decreased with further increases in temperature. Given that in some cases only two data points (at 23 and 25 °C) were available for analysis for the 23 °C-acclimated abalone, these groups were not analysed further. Thus, rate increases were analysed only for 16 °C-acclimated abalone at temperatures between 17 and 25 °C.

Table 6.1: Regression equations for temperature (temp) versus heart rate or oxygen consumption rate ($\dot{M}O_2$), Pearson's correlation coefficients (r) for heart rate (HR) and $\dot{M}O_2$ (* = $p < 0.01$), and Q_{10} values of *H. rubra*, *H. laevigata*, and hybrids during exposure to increasing temperatures from 17 to 25 °C. Abalone were acclimated to 16 °C and different dissolved oxygen levels [% air saturation (O_2sat)]. Mean ± SE. n = 9 to 12.

		Equation			**Q_{10}**	
Type of abalone	**Oxygen level**	Heart rate	$\dot{M}O_2$	R	Heart rate	$\dot{M}O_2$
Hybrid	100	HR= temp × 3.49 - 23.90	$\dot{M}O_2$= temp × 0.22 - 2.35	+0.867*	1.99±0.07	2.66±0.24
	70	HR= temp × 2.94 - 12.33	$\dot{M}O_2$= temp × 0.18 - 1.70	+0.873*	1.81±0.08	2.52±0.23
	70/100	HR= temp × 2.56 - 4.39	$\dot{M}O_2$= temp × 0.19 - 1.64	+0.854*	1.74±0.03	2.44±0.14
H. rubra	100	HR= temp × 2.90 - 12.58	$\dot{M}O_2$= temp × 0.20 - 1.76	+0.845*	1.95±0.13	2.35±0.11
	70	HR= temp × 3.21 - 13.52	$\dot{M}O_2$= temp × 0.19 - 2.17	+0.686*	2.14±0.14	2.89±0.32
	70/100	HR= temp × 2.20 + 8.96	$\dot{M}O_2$= temp × 0.17 - 0.89	+0.796*	1.61±0.05	1.93±0.10
H. laevigata	100	HR= temp × 3.04 - 20.95	$\dot{M}O_2$= temp × 0.17 - 1.71	+0.676*	2.08±0.10	2.73±0.23
	70	HR= temp × 3.00 - 14.06	$\dot{M}O_2$= temp × 0.18 - 1.58	+0.717*	1.87±0.06	2.65±0.28
	70/100	HR= temp × 2.45 - 1.95	$\dot{M}O_2$= temp × 0.15 - 1.05	+0.770*	1.62±0.09	2.08±0.18

N.B. 70/100 = abalone acclimated to 70% O_2sat and measured in 100% O_2sat.

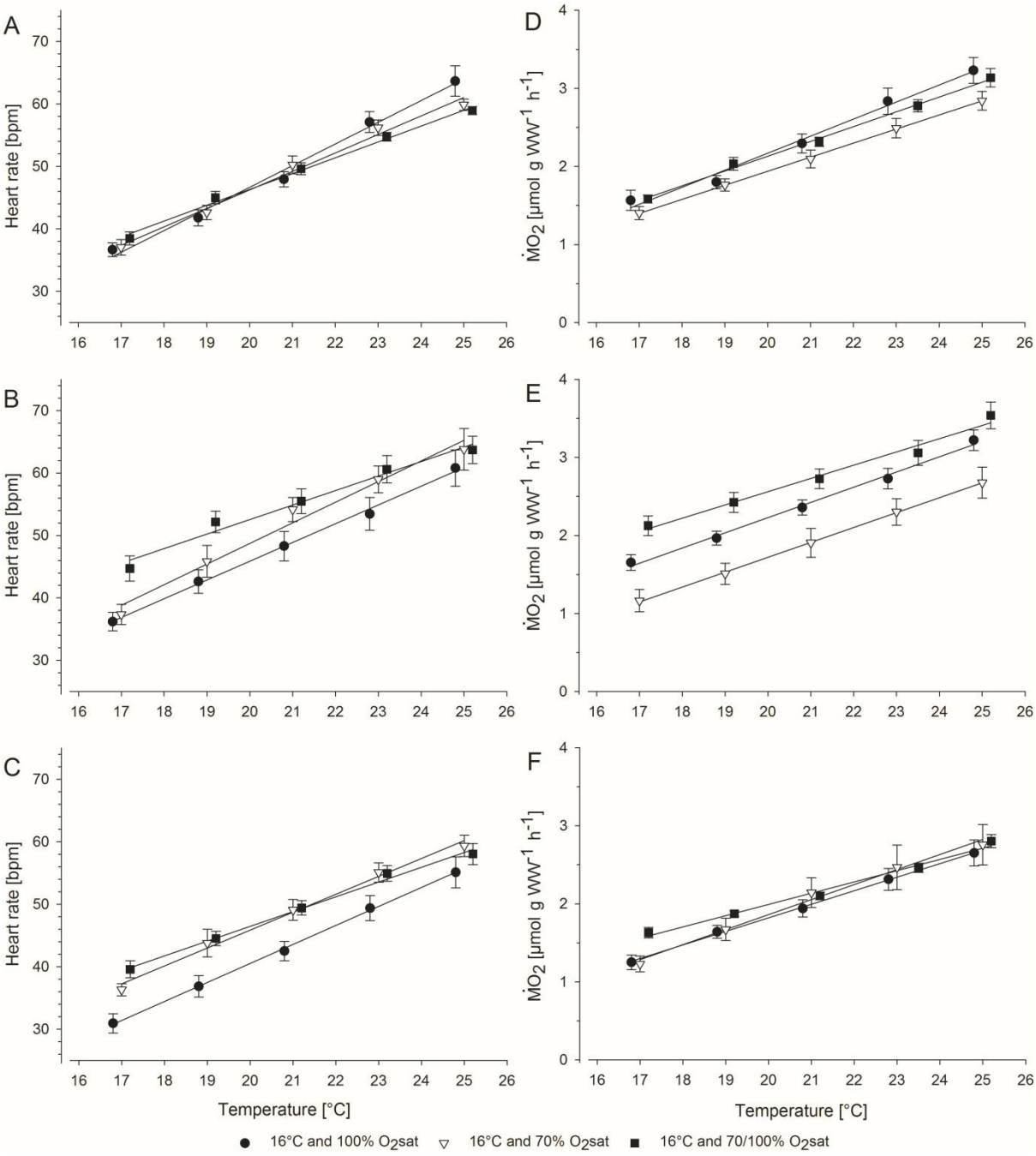

Fig. 6.3: Heart rate [bpm] (A–C) and oxygen consumption rates ($\dot{M}O_2$) [µmol g WW^{-1} h^{-1}] (D–F) of hybrid abalone (A, D), *H. rubra* (B, E), and *H. laevigata* (C, F) exposed to increasing temperatures from 17 to 25 °C. Abalone were acclimated to 16 °C and 100% air saturation (O_2sat) (closed circles), 70% O_2sat (open triangle) and acclimated to 70% but measured at 100% O_2sat (closed square). Symbols are offset for visual clarity of error bars. Equations for regression lines are stated in Table 6.2. n = 9 to 12.

Heart rate

The influence of experimental temperature on heart rate was similar for all three types of abalone in that increasing temperatures from 17 to 25 °C resulted in a 1- to 2-fold increase in heart rate (Fig. 6.3A-C). Further, a significant interaction between oxygen level and experimental temperature was seen in all three types of abalone. For hybrids, exposure to an acute increase in oxygen (i.e. from 70% to 100% O_2sat) resulted in a significantly higher heart rate/temperature intercept in comparison to hybrid$_{16C_100}$ ($p < 0.0001$) (Fig. 6.3A). Yet, heart rate/temperature intercepts for *H. rubra*$_{16C}$ and *H. laevigata*$_{16C}$ under increased oxygen were significantly higher than those of both 100% O_2sat ($p < 0.02$) and 70% O_2sat ($p < 0.02$) acclimated individuals (Fig. 6.3B and C). Regression equations for heart rate/temperature slopes for all acclimation conditions and types of abalone are given in Table 6.1.

Oxygen consumption rate

Acclimation condition had a different effect on $\dot{M}O_2$ between types of abalone. For all hybrid$_{16C}$ abalone, $\dot{M}O_2$ doubled during exposure to increasing experimental temperatures from 17 to 25 °C ($p < 0.001$) (Fig. 6.3D; Table 6.1). Oxygen level had no influence on the relationship. This result was similar to that observed for *H. laevigata*$_{16C}$ (Fig. 6.3F), but different to that for *H. rubra*$_{16C}$ (Fig. 6.3E). Oxygen level had a significant influence on the $\dot{M}O_2$/temperature slope of *H. rubra*$_{16C}$ ($p = 0.001$). The intercept was significantly higher in *H. rubra*$_{16C\_70/100}$ ($p < 0.05$) and lower in *H. rubra*$_{16C\_70}$ ($p < 0.02$) compared to *H. rubra*$_{16C\_100}$ (Fig. 6.3E). Regression equations for $\dot{M}O_2$/temperature slopes for all acclimation conditions and types of abalone are given in Table 6.1.

Arrhenius break-points

Arrhenius break-point temperatures

For hybrids, ABTs of heart rate and $\dot{M}O_2$ were generally higher in 23 °C-acclimated abalone compared with 16 °C-acclimated abalone, a pattern that was similar to *H. laevigata*, but different to *H. rubra* (Fig. 6.4; Table 6.2). Arrhenius break-point temperatures of heart rate and $\dot{M}O_2$ in hybrid$_{23C_100}$ and hybrid$_{23C_70}$ were between 28 and 29 °C and those of hybrids at all other acclimation groups were between 26 and 28 °C. The difference was not statistically significant between acclimation groups within hybrids ($p > 0.05$) (Fig. 6.4A; Table 6.2). Yet, the heart rate ABTs of hybrid$_{23C_100}$ and hybrid$_{23C_70}$ were significantly higher in comparison with those of *H. laevigata*$_{23C\_100}$ and *H. laevigata*$_{23C\_70}$ ($p < 0.001$) (Fig. 6.4A-C; Table 6.2).

For *H. laevigata*$_{23C}$, heart rate and $\dot{M}O_2$ ABTs were between 27 and 29 °C and lower in *H. laevigata*$_{16C}$ with values between 26 and 27 °C. The differences were significant between *H. laevigata*$_{23C\_100}$ and *H. laevigata*$_{16C\_100}$ ($p < 0.001$) as well as *H. laevigata*$_{16C\_70}$ ($p < 0.02$) (Fig. 6.4C; Table 6.2).

In contrast to hybrids and *H. laevigata*, higher acclimation temperatures did not result in higher ABTs for *H. rubra* (Fig. 6.4; Table 6.2). Arrhenius break-point temperatures for $\dot{M}O_2$ were similar between all acclimation conditions and ranged from 25 to 28 °C (Fig. 6.4B; Table 6.2). Heart rate ABTs ranged between 26 and 27 °C with significantly lower values in *H. rubra*$_{16C\_70/100}$ ($p < 0.005$) and *H. rubra*$_{23C\_70}$ ($p < 0.01$) in comparison to *H. rubra*$_{16C\_100}$ (Fig. 6.4B; Table 6.2).

Heart rate and $\dot{M}O_2$ maxima

Hybrids had similar $\dot{M}O_2$ maxima (average 3.3 ± 0.1 µmol gWW^{-1} h^{-1}) and heart rate maxima (average 61.9 ± 0.8 bpm) irrespective of acclimation oxygen level and temperature ($p > 0.05$) (Fig. 6.4D; Table 6.2), which was in contrast to both pure species. In general, heart rate maxima of *H. rubra*$_{23C}$ and *H. laevigata*$_{23C}$ tended to be ~10% lower in comparison to *H. rubra*$_{16C}$ and *H. laevigata*$_{16C}$ (Fig. 6.4E and F; Table 6.2). Yet, the difference was only statistically significant between *H. rubra*$_{23C\_70}$ and *H. rubra*$_{16C\_70/100}$ ($p < 0.002$) (Fig. 6.4E; Table 6.2). Maximum $\dot{M}O_2$ values showed a different trend between the two pure species in that *H. rubra* had highest $\dot{M}O_2$ maxima when acclimated to 16 °C but *H. laevigata* had highest $\dot{M}O_2$ maxima when acclimated to 23 °C (*H. rubra*$_{16C\_100}$ = 3.8 ± 0.1 µmol gWW^{-1} h^{-1}, *H. laevigata*$_{23C\_100}$ = 4.0 ± 0.2 µmol gWW^{-1} h^{-1}) (Fig. 6.4E and F; Table 6.2).

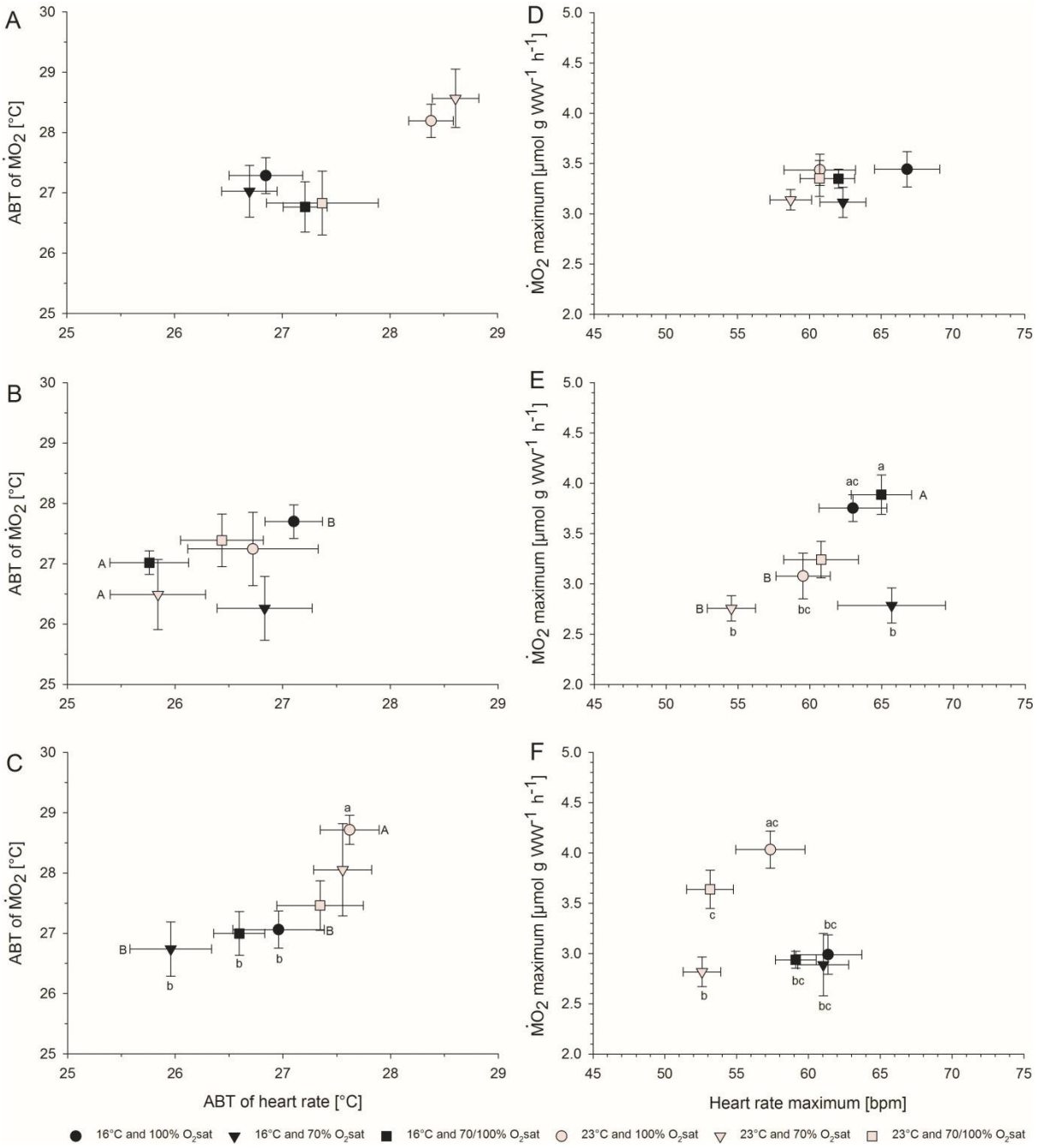

Fig. 6.4: Arrhenius break-point temperatures (ABT) [°C] (A–C) and maximum values for oxygen consumption rate ($\dot{M}O_2$) [µmol g WW^{-1} h^{-1}] and heart rate [bpm] (D–F) of hybrid abalone (A, D), *H. rubra* (B, E), and *H. laevigata* (C, F) acclimated to 16 °C (closed symbols) and 23 °C (open symbols) at 100% O_2sat (circles), 70% O_2sat (triangles) and 70% O_2sat but measured in 100% O_2sat (squares). Different *upper case letters* indicate significant differences of heart rate measures between acclimation conditions. Different *lower case letters* indicate significant differences of $\dot{M}O_2$ measures between acclimation conditions. Mean ± SE. n = 9 to 12.

Table 6.2: Arrhenius break-point temperatures (ABT) [°C] and maximum values for heart rate [bpm] and oxygen consumption rate ($\dot{M}O_2$) [µmol g WW^{-1} h^{-1}] of *H. rubra*, *H. laevigata*, and hybrids acclimated to two temperatures [°C] and three dissolved oxygen conditions [% air saturation (O_2sat)]. Mean ± SE. n = 9 to 12.

	Acclimation		ABT		Maximum value	
Species	**Temperature (°C)**	**Oxygen level (% O_2sat)**	**Heart rate (°C)**	**$\dot{M}O_2$ (°C)**	**Heart rate (bpm)**	**$\dot{M}O_2$ (µmol g WW^{-1} h^{-1})**
Hybrid	16	100	26.8±0.4	27.3±0.3	67.8±2.3	3.4±0.2
		70	26.7±0.3	27.0±0.4	62.3±1.6	3.1±0.1
		70/100	27.2±0.2	26.8±0.4	62.0±1.1	3.3±0.1
	23	100	28.4±0.2	28.2±0.3	60.7±2.5	3.4±0.2
		70	28.3±0.3	28.6±0.5	59.7±1.7	3.1±0.1
		70/100	27.4±0.5	26.8±0.5	60.7±1.4	3.4±0.2
H. rubra	16	100	27.1±0.3	27.7±0.3	63.0±2.4	3.8±0.1
		70	26.8±0.4	26.3±0.5	65.7±3.8	2.8±0.2
		70/100	25.8±0.4	27.0±0.2	65.0±2.1	3.9±0.2
	23	100	26.7±0.6	27.0±0/6	59.5±1.9	3.0±0.2
		70	25.8±0.4	26.4±0.5	54.5±1.7	2.6±0.1
		70/100	26.4±0.4	27.4±0.4	60.8±2.6	3.2±0.2
H. laevigata	16	100	27.0±0.4	27.1±0.3	61.4±2.4	3.0±0.2
		70	26.0±0.4	26.4±0.5	61.0±1.8	2.9±0.3
		70/100	26.6±0.2	27.0±0.4	59.1±1.4	2.9±0.1
	23	100	27.6±0.2	28.5±0.3	55.6±2.2	3.9±0.2
		70	27.6±0.3	28.1±0.8	52.6±1.3	2.8±0.1
		70/100	27.3±0.4	27.6±0.4	53.1±1.6	3.6±0.2

N.B. 70/100 = abalone acclimated to 70% O_2sat and measured in 100% O_2sat.

Discussion

The present study was conducted to determine behavioural and physiological responses of hybrid abalone that may explain their growth heterosis in comparison to the pure parental species. Movement of hybrids and *H. laevigata* was not affected by oxygen levels and temperature, while *H. rubra* showed a strong thermal response (Fig. 6.2). Heart rate and $\dot{M}O_2$ of hybrids remained similar irrespective of oxygen level while pure species adjusted both parameters (Fig. 6.3). Arrhenius break-point temperatures of hybrids and *H. laevigata* were higher when acclimated to 23 °C compared with 16 °C which was not observed for *H. rubra*

(Fig. 6.4A-C). When comparing maximum heart rate and $\dot{M}O_2$ values, those of hybrids were more stable than those of both pure species (Fig. 6.4D-F). While the differences detected between hybrids and pure species were minor, they do support the original hypothesis that hybrids are less sensitive to changes in environmental conditions. This tendency for environmental resilience may ultimately contribute to the growth advantage of hybrids over long grow-out periods in the environmentally unstable aquaculture environment. It should be considered, however, that abalone used in the present study were sourced from one family; this minimises genetic variation when comparing across treatments, but does not allow assessments of the breadth of variability across the broader population. Presumably the advantages must be limited in the natural environment because hybrid prevalence is low.

Movement in gastropods is energetically expensive due to the high costs associated with crawling and mucous production (Denny, 1980; Werner *et al*., 1995; Donovan and Carefoot, 1997; Robinson *et al*., 2013). Therefore, it is assumed to mainly occur because of disturbance or to find food (Werner *et al.*, 1995; Robinson *et al*., 2013). Since food was not provided during this experiment, the differences in movement are likely to be associated with the treatment challenges. Increased movement as a response to thermal stress requires that energy is directed towards muscular work and mucous production and away from growth. Thus, the consistent shorter distance travelled across temperatures in hybrids may translate to a growth advantage under fluctuating thermal conditions compared with *H. rubra* but not compared with *H. laevigata* (Fig. 6.2). Without thermal stress, longer distances travelled can be beneficial to exploit resources and can overrule the costs of transport (Cenni *et al.*, 2009). This may lead to a growth advantage for the hybrid in comparison to the more stationary *H. laevigata*. These behaviours may be particularly beneficial in artificial environments, where abalone do not have to forage because sinking food pellets are used in high quantities and negative effects of movement such as a higher risk of predation due to higher visibility are minimised (Cenni *et al*., 2009). Indeed, cultured hybrid abalone have a higher food intake than *H. laevigata* (Currie *et al*., 2016). Hence, the longer distance travelled in hybrids may add to their growth advantage in comparison to the slower growing and less active *H. laevigata*.

Hybrid abalone heart rate and metabolic rate were consistently more stable across temperatures and oxygen saturations than the pure species, suggesting that hybrids are less sensitive to changes in oxygen level. In addition, the higher ABTs in hybrids (and *H. laevigata*) acclimated to 23 °C versus those acclimated to 16 °C suggest that they were better

able to acclimate to the higher experimental temperature compared to *H rubra*. In combination, these results may illustrate an energetic advantage for the hybrid and ultimately contribute to higher growth. First, sensitivity to oxygen is energetically expensive because of long-term costs associated with repair and replacement of oxygen damaged macromolecules (Hawkins and Day, 1999; Somero, 2002). Hence, the protein turnover is reduced in less sensitive individuals, which leads to lower energy requirements and results in a greater potential for growth (Toro *et al*., 1996; Hawkins and Day, 1999). Second, thermal acclimation leads to adjustments on the cellular level which are initially costly but ultimately result in a shift of the optimum and maximum performance temperatures. As a consequence, more energy is available in the long term for growth under the prevailing thermal conditions (Newell and Kofoed 1977; Beiras *et al*., 1995; Sokolova *et al*., 2012).

The constant heart rate and $\dot{M}O_2$ at different oxygen levels in hybrids does not necessarily indicate that hybrids were not responding to oxygen levels. In *H. iris*, for example, heart rate also remained stable after exposure to environmental hypoxia (Ragg and Taylor, 2006a). Nevertheless, it was shown that blood flow in *H. iris* increased 2.7-fold above rates of unstressed individuals in normoxia and it was hypothesised that this resulted from an increased stroke volume (Ragg and Taylor, 2006a). Blood flow was not measured in the present study, but it is possible that elevated stroke volume contributed to the more stable heart rate seen in hybrids. Yet, it was further suggested by Ragg and Taylor (2006a) that the increase in blood flow seen in *H. iris* would promote an increase in $\dot{M}O_2$, which was not the case for hybrids in the present study. Future work, including simultaneous measurements of heart rate, cardiac output, and $\dot{M}O_2$ could clarify whether hybrids adjust stroke volume to maintain $\dot{M}O_2$ at varying oxygen levels or if they are indeed unaffected by the oxygen levels used here.

Hybrids and *H. laevigata* but not *H. rubra* showed thermal acclimation capacity. The ability to thermally acclimate is strongly dependent on genetics and correlates with the biogeographical distribution in congeners of abalone (Dahlhoff and Somero, 1993; Liang *et al*., 2014). For example, five American abalone species could only acclimate their mitochondrial respiration when exposed to temperatures that they commonly experience in nature. The five species did not acclimate to temperature extremes that occur in their full biogeographical distribution (Dahlhoff and Somero, 1993). In line with these observations, it is possible that *H. rubra* was not able to acclimate to 23 °C because this is outside the temperature range where this species is commonly found - 11 and 19 °C (Shepherd, 1973). In

contrast, *H. laevigata* commonly experiences temperatures up to 23 °C, which may have enabled it to acclimate to this temperature in the present study. The fact that the hybrid also showed acclimation capacity at 23 °C suggests that it may have inherited the heat tolerance trait of *H. laevigata*. Similar results were observed in cultured Chinese abalone, in which the interspecies hybrid between *H. discus hannai* and *H. gigantea* inherited the thermal tolerance trait of the more heat tolerant *H. gigantea* (Liang *et al*., 2014).

Arrhenius break-point temperatures for heart rate and $\dot{M}O_2$ varied by 4 °C, between 25 and 29 °C, between types of abalone in this study. While there is some evidence from other studies that thermal tolerance of abalone is size dependent (Steinarsson and Imsland, 2003; Searle *et al*., 2006), the size range used in the present study spanned only 61 to 67 mm SL, precluding a comprehensive examination of the influence of size on thermal tolerance. The variation of 4 °C in ABTs between types of abalone in this study is greater than the slightly different upper critical temperatures reported previously for the pure species, *H. rubra* - 26.9 °C and *H. laevigata* - 27.5 °C, measured as the temperature at which the abalone detached from the substrate (Gilroy and Edwards, 1998). This suggests that ABTs for heart rate and $\dot{M}O_2$ may represent more sensitive measures of heat tolerance in abalone compared with upper critical temperature measurements. This conclusion is in accordance with similar observations between heart rate ABTs and traditional upper critical temperature experiments in Chinese abalone, *H discus hannai*, *H. gigantea*, and their interspecies hybrid (Chen *et al*., 2016). Further, the ABTs determined for abalone in the present study are only 2 to 6 °C above summer sea temperatures of 23 °C occurring in natural habitats of their founders, rendering abalone, especially *H. rubra*, vulnerable to future climate change-induced temperature increases (Fordham *et al.*, 2013).

In summary, this study suggests that *H. rubra* × *H. laevigata* hybrids maintain their $\dot{M}O_2$, heart rate, and movement over a broader environmental range compared with their parental pure species. Maintaining optimal physiological rates during environmental fluctuations may contribute to the hybrid growth advantage in the environmentally unstable aquaculture environment. Thus, future plans by aquaculture farmers to increase oxygen levels in grow-out tanks on their farms may be more beneficial for pure species than for hybrids. This suggestion should be considered with care and long-term investigations of growth rates and energetics of pure and hybrid abalone at varying and stable environmental conditions should be conducted to further improve the understanding of the apparent growth advantage in

hybrids. While the present study provides some evidence for hybrid vigour in farmed abalone, it remains unclear why the prevalence of hybrids is low in nature.

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CHAPTER 7: General discussion

Results of this thesis have improved the current understanding about environmental impacts on the physiology and behaviour of cultured abalone and the findings can be divided into two main components. First, determined physiological and behavioural changes across development of hybrids raise questions about common aquaculture practices. Second, identified superior physiological and behavioural performances of hybrids in comparison to parental pure species, *H. rubra* and *H. laevigata*, suggest that the hybrid is better suited for culture at the aquaculture farm where they were sourced from, i.e. JTA.

Ontogenetic changes in tolerance to temperature and oxygen of hybrids

Studies within this thesis indicated that the thermal tolerance of the hybrid is largely conserved across tested ages. The temperature at which abalone $\dot{M}O_2$ reached a maximum was 25 °C in larvae (Chapter 4) and 27 °C in 22 month old hybrids (Chapter 6). Hooper *et al*. (2014) demonstrated that 2 year old *H. rubra* × *H. laevigata* hybrids originating from the same latitude than hybrids used in the current study were also able to withstand 26 °C for 7 days. A higher termpertature was not tested by Hooper *et al*. (2014). In combination with results of this thesis these results indicate that the thermal tolerance of hybrids does at least not decrease with an increasing age up to two years. Yet, it was also shown that exposure of 2 year old hybrids to 26 °C for 7 days lead to sub-lethal immunologic effects and damaged gill as well as digestive gland tissue rendering individuals more susceptibe to mortality during summer (Hooper *et al*., 2014). In this thesis, short-term responses of hybrids were tested and it is now time to extend and validate the thermal tolerance of hybrids during exposure to high and fluctuating temperatures for extended periods of time. With this information it would be able to predict survival rates on aquaculture farms during summer months, the ultimate information invaluable for abalone farmers. At JTA, the grow-out out phase can last for up to two years and survival experiments should be conducted with several age/size classes. Experiments with *H. laevigata* originating from similar latitudes than abalone used in the present thesis, for example, have demonstrated that exposure to temperatures of 25 to 26 °C for extended periods of time (> 30 days) caused increased mortalities in comparison to exposure to 22 °C (Lange *et al*., 2014; Stone *et al*., 2014; Duong *et al*., 2016). The increased mortality, however, was only recorded for three year old *H. laevigata*, while survival rates of 2 year old individuals were not different between 22 and 26 °C (Stone *et al*., 2014). Dietary

intervention increased survival rates of three year old *H. laevigata* during exposure to high temperatures (Lange *et al*., 2014; Stone *et al*., 2014; Duong *et al*., 2016). Although hybrids are usually harvested at a younger age than 3 years at JTA, diet supplementation is an interesting field of study with the potential to further improve the health of cultured hybrids.

In addition, long-term thermal tolerance studies should be carried out not only for grow-out phase individuals but also with nursery stage abalone. Future plans of some Australian abalone farmers include a temperature-controlled nursery posing the opportunity to create environmental conditions that promote maximal growth rates. Increasing the temperature in the nursery within the ecological temperature range of abalone is likely to promote faster growth (Angilletta *et al.*, 2004). Yet, the nursery stage is long enough to potentially contain varied thermal optima for growth. It is possible that the thermal preferences of nursery-stage abalone, similar to thermal preferences of larvae in this thesis (Chapter 4), change across development as demonstrated for *H. rufescens* (Steinarsson and Imsland, 2003) and *H. iris* (Searle *et al.*, 2006). Indeed, differences in temperature dependent growth rate across age classes have also been demonstrated for *H. laevigata* and thus may be apparent for the *H. rubra* × *H. laevigata* hybrid as well. Growth rates, measured as biomass gain, in 1 year old *H. laevigata* doubled when temperature was increased from 14 to 18 °C and further increased by 25% when temperature was increased to 22 °C (Stone *et al.*, 2013). Yet, biomass gain of 2 year old *H. laevigata* increased with temperature from 14 to 18 °C but no significant biomass gain was reached with exposing individuals to a higher temperature of 22 °C (Stone *et al.*, 2013). A comprehensive study with several size classes of nursery-stage abalone should be investigated to determine temperatures at which growth of abalone is maximal to prevent revenue loss due to e.g. heating expenses. Further, a classic therory, termed the Bergmann's rule, states that animals inhabiting a large geographical range exhibit larger body sizes in colder environments (reviewed by Blackburn *et al.* (1999)). Generally, in ectotherms, exposure to higher temperatures results not only in faster growth but also in reaching maturity at a smaller size (Angilletta *et al.*, 2004). If this is the case also for abalone, then individuals direct energy towards reproduction at a smaller size which causes growth rates to slow down, with a consequent reduction in adult size. Thus, experiments with nursery-stage abalone that were exposed to higher temperatures should be followed up until harvest to determine if nursery heating will increase farm production.

Comparisons of hypoxia tolerances revealed drastic changes across hybrid life-stages. Fertilized eggs and larvae of hybrids maintained their $\dot{M}O_2$ over a wide oxygen range, i.e. P_{crit}

levels ranged between 14 and 23% O_2sat in these early life-stages (Chapter 3). Yet, 15 month old hybrids regulated $\dot{M}O_2$ only down to 40% O_2sat (Chapter 5) and 2 year old hybrids had poor hypoxia tolerance with a P_{crit} of only 80% O_2sat (A. Morash, Mount Allison University, unpubl. data, 2014). Changes in tolerance to low oxygen levels during development has also been demonstrated for other species (De Silva and Tytler, 1973; Ishibashi *et al*., 2005; Ishibashi *et al*., 2007; Alter *et al*., 2015). Information about P_{crit} levels across life stages in abalone is of high importance because control of oxygen on abalone farms decreases as abalone age, exposing increasingly sensitive individuals to higher fluctuations in oxygen levels. During decreasing oxygen levels juvenile abalone increasingly rely on the less efficient anaerobic energy production even at oxygen levels above their P_{crit}, thus lowering the potential for growth (Chapter 5). Inded, extended exposure of *H. laevigata* to oxygen levels below their P_{crit} has been demonstrated to significantly reduce growth either measured by SL or whole body weight (Harris *et al*., 1999). The dependence on anaerobic energy pathways in abalone from the present thesis was accentuated during exposure to an additional stressor, i.e. high temperature (Chapter 5). On aquaculture farms, juveniles are likely to encounter further environmental stressors, such as variation in salinity, increased CO_2 and associated decreases in pH, as well as farm specific stressors, such as high stocking densities and nitrogenous waste. These additional stressors result in increased metabolic constraints and thus reduced growth potential (reviewed in Chapter 2). It should be noted that juveniles used for experiments in this thesis were acclimated to stable environmental conditions, which is different to the fluctuating abiotic farm environment and hence may have influenced tolerance limits of abalone. One way to address both issues (i.e. synergistic effects of additional farm stressors and stable laboratory conditions) is to monitor sentinel abalone directly in the farm environment. A recently developed heart rate sensor (UTas/CSIRO) was successfully tested in laboratory experiments with abalone (Chapter 6) and has laid the foundation for such real-time analyses on abalone farms (c.f. Andrewartha *et al.*, 2015). Some aquaculture farmsers explore the possibility to supplement also grow-out tanks with oxygen to reduce one farm stressor with an aim to increase growth rates (A. Krisnich, JTA, pers. comm., December 2016). Sensor technology in combination with oxygen injection systems has not only been demonstrated to improve growth in cultured fish but it was also economically viable with benefits of improved growth of stock exceeding the costs of oxygen supplementation (Bergheim *et al*., 2006).

Hybrid vigour

Finding underlying mechanisms for the improved growth of hybrids was the main driver of this study and is of high interest for the abalone industry which has an overarching goal to further improve growth rates of abalone. It was expected that the hybrid has a higher growth rate when compared to parental species because the hybrid A) is less active, B) is less sensitive to fluctuations in abiotic factors and/or C) has a higher metabolic rate and thus gains more energy per time unit ("increased intake hypothesis"). Advantages of hybrids were not observed in the hatchery phase (Chapter 4) and became apparent in juvenile life-stages (Chapter 6). This finding is in accordance with growth rates which are also only higher in juvenile hybrids when compared with pure species (A. Krsinich, JTA, pers. comm., November 2013). In the earliest life stages, swimming speed and $\dot{M}O_2$ of hybrids were similar to those of pure species at temperatures commonly experienced on aquaculture farms. Yet, at 25 °C, increasing swimming speeds across larval development and higher $\dot{M}O_2$ was observed in hybrids and *H. rubra* in comparison to *H. laevigata*. It is likely that both differences are not an indication of hybrid vigour, but instead they are a result of maternal influences. Indeed, high influence of maternal factors affecting the swimming speed in veligers has also been reported for the common slipper snail, *Crepidula fornicata* (Hilbish *et al.*, 1999). Hybrid larvae may closely resemble *H. rubra* because they were produced via *H. rubra* eggs and thus hybrids inherit *H. rubra* mitochondria in which the oxygen consumed is utilised in the production of energy (ATP) for swimming. To date, no other study has quantified physiological or behavioural differences between early life stages of pure parental abalone and their hybrids. Yet, studies on fertilization and survival rates of early life abalone hybrids are abundant (Leighton and Lewis, 1982; Hahn, 1989; Hoshikawa *et al*., 1998; Wang and Fan, 1999; Cai *et al*., 2006; Ahmed *et al*., 2008b; Luo *et al*., 2010; Lafarga-de la Cruz *et al*., 2013). Varying development time between early life stages of pure and hybrid abalone can be used as an indicator for metabolism. In agreement to our study, no difference in terms of developmental time has been found in the early-life stages of *H. rufescens*, *H. discus hannai*, and their interspecies hybrid (Lafarga-de la Cruz *et al*., 2013). These individuals were monitored until an age of 15 months, at which, similar to the present thesis, juvenile hybrids were not significantly different in SL in comparison to their parental species. Yet, juvenile hybrids had a significantly higher thermal tolerance in comparison to their pure parental species potentiallly indicating that hybrid vigour in *H. rufescens* × *H. discus hannai*, similar to heterosis in *H. rubra* × *H. laevigata*, becomes aparent later in life (Lafarga-de la Cruz *et al*., 2013).

At 15 months of age (Chapter 5), the differences in physiological and behavioural responses to typical farm conditions between the hybrid and both pure species were indicative of hybrid vigour, yet results provided little evidence for the three hypotheses. The lack of outstanding physiological differences in 15 months old abalone matched their size because hybrids were only slightly larger than *H. rubra* and showed no size difference in comparison to *H. laevigata* at this age. Yet, the resting $\dot{M}O_2$ of hybrids tended to be lower in comparison to both pure species but this was only significant in one out of four environmental treatments tested. This may give some support for the compensation hypothesis which predicts that individuals with lower resting $\dot{M}O_2$, due to lower maintenance costs, can channel more energy into growth (reviewed by Burton *et al*. (2011)). In aggreement with this hypothesis are results of studies conducted with oysters that were selected for increased growth (reviewed by Bayne (2004)). In comparison to control individuals, fast-growing individuals had reduced metablic rates as well as reduced metabolic costs for growth which were achieved by a more efficient protein turnover (reviewed by Bayne (2004), c.f. Chapter 6). Further, fast-growing oysters attained their increased growth also partly by a higher food intake and a different feeding behaviour in comparison to control individuals (reviewed by Bayne (2004)). Similar results have been found for other heterozygous mussels and fish in which fast-growing individuals had reduced maintenance costs and increased food intake (Hawkins *et al*., 1986; Present and Conover, 1992; Bayne and Hawkins, 1997; Thodesen *et al*., 1999). Behavioural differences between *H. rubra* × *H. laevigata* hybrids and their parental species were minimal in 15 months old individuals but became more apparent in 22 months old individuals (Chapter 5 and 6). During experiments in this thesis food was not offered, yet a recent laboratory study by Currie *et al*. (2016) demonstrated that 18 months old *H. rubra* × *H. laevigata* hybrids have indeed a higher food intake in comparison to *H. laevigata* (food intake of the parental species *H. rubra* was not determined). It would be interesting to investigate behaviour and food intake of both parental species and their hybrid in an aquaculture setting to validate if the apparent growth of hybrids is indeed at least partly due to increased feeding rates.

At 22 months of age (Chapter 6) the hybrid had grown to a larger size than both parental species. At this stage, the behavioural and physiological responses of the hybrid to typical farm environmental stressors differed to those of parental pure species supporting the hypothesis that the hybrid is less sensitive to fluctuations in abiotic factors than pure species. The tendency for environmental resilience in 22 month old hybrids may ultimately contribute

to their growth advantage over long grow-out periods in the environmentally unstable aquaculture environment. One major concern of abalone farmers is summer mortality, a condition that is partly caused by exposure of abalone to either spikes or extended time periods of high temperatures (Handlinger *et al.*, 2005). In Chapter 6, it was demonstrated that the hybrid has a higher heat tolerance in comparison with parental pure species, which is in accordance with results from *H. rufescens* × *H. discus hannai* hybrids (Lafarga-de la Cruz *et al*., 2013). Other hybrids have been reported to inherit the thermal tolerance of one parental species (Hoshikawa *et al*., 1998; Liang *et al*., 2014). The heat tolerance of *H. discus hannai* × *H. gigantea* hybrids was higher in comparison to *H. discus hannai*, but similar to that of *H. gigantea* (Liang *et al*., 2014). Hybrids between *H. discus hannai* × *H. kamschatkana* selected for cold tolerance inherited the thermal tolerance of the more cold tolerant *H. kamtschatkana* (Hoshikawa *et al.*, 1998). The present thesis, however, demonstrated heterosis for heat tolerance but also heterosis for oxygen tolerance (Chapter 6) a trait that has not been previously reported for abalone hybrids and is probably beneficial for growth in the oxygen-unstable grow-out environment on aquaculture farms.

Differences in physiological and behavioural responses to environmental factors between hybrids and pure species were minor. It appears that the growth advantage of hybrid abalone is attained by a series of small energetically advantageous alterations, which has also be concluded for other heterozygotous molluscs (Bayne, 2004). In this regard, another factor, shell growth, which has not been mentioned in any previous chapter, may also contribute to the improved hybrid growth. In Fig. 7.1 the size scaling of shell mass of the three types of abalone used in this thesis is shown. Across all juvenile sizes, the hybrid had an intermediate shell/body mass ratio (0.514 ± 0.005) between the lower ratio for *H. rubra* (0.454 ± 0.005) and the higher ratio for *H. laevigata* (0.605 ± 0.007). A decreased cost of shell production in hybrids in comparison to *H. laevigata* may be an additional minor alteration that contributes to the growth advantage of the hybrid. Similar results have been shown for *Helix aspersa* Müller snails that were selected for fast growth. Fast-growing individuals produced lighter shells in comparison to slow-growing individuals and it was concluded that superior tissue growth is partly achieved at the expense of shell production (Czarnolęski *et al.*, 2008).

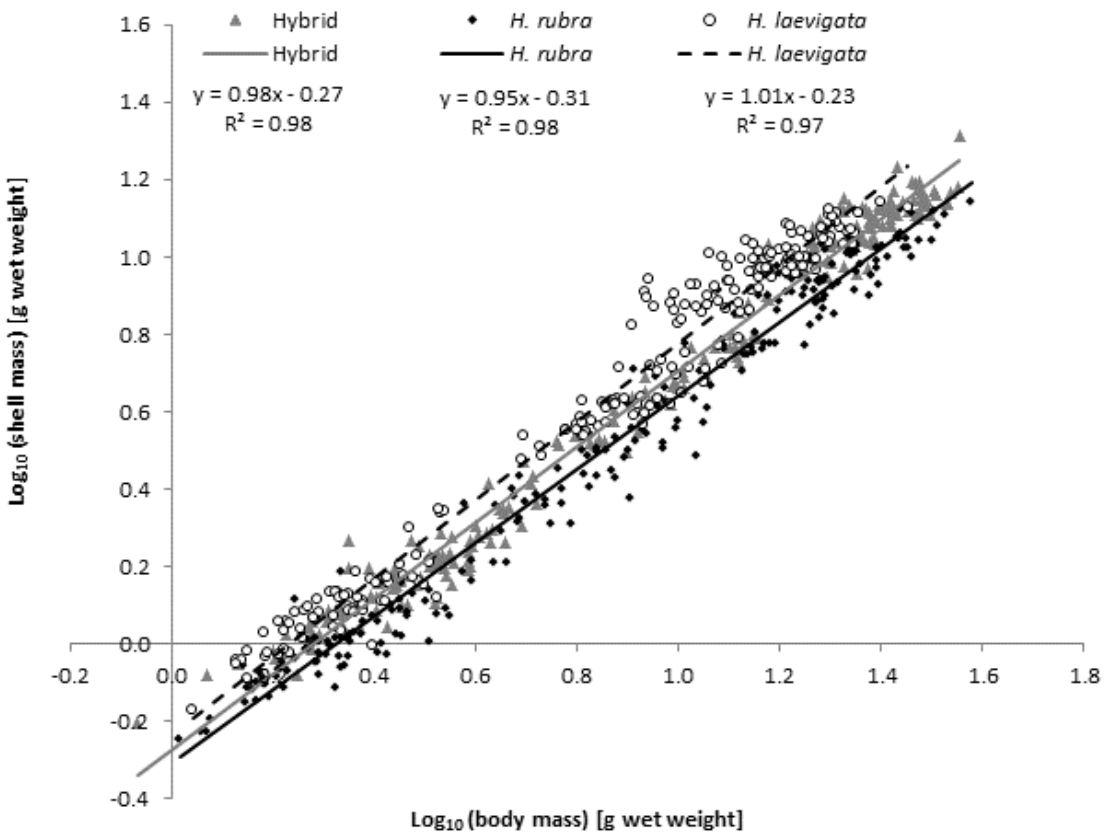

Fig. 7.1.: Size scaling of shell mass of hybrids (grey triangle, grey line), *H. rubra* (black diamonds, black line), and *H. laevigata* (open circles, dashed line). Symbols represent individual juvenile abalone used for experiments within this thesis.

This thesis has taken significant steps to understand physiological and behavioural attributes that contribute to the growth advantage of hybrid abalone. Yet, much work remains to be done to uncover the elusive mechanisms driving hybrid vigour.

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